

HYBRID PROTEINS WHICH FORM HETERODIMERS

FIELD OF THE INVENTION

5 The present invention relates to a hybrid protein comprising two coexpressed amino acid sequences forming a dimer, each comprising:

10 a) at least one amino acid sequence selected from a homomeric receptor, a chain of a heteromeric receptor, a ligand, and fragments thereof; and

15 b) a subunit of a heterodimeric proteinaceous hormone or fragments thereof; in which (a) and (b) are bonded directly or through a peptide linker, and, in each couple, the two subunits (b) are different and capable of aggregating to form a dimer complex.

BACKGROUND OF THE INVENTION

20 Protein-protein interactions are essential to the normal physiological functions of cells and multicellular organisms. Many proteins in nature exhibit novel or optimal functions when complexed with one or more other protein chains. This is illustrated by various ligand-receptor combinations that contribute to regulation of cellular activity. Certain ligands, such as tumor necrosis factor α (TNF α), TNF β , or
25 human chorionic gonadotropin (hCG), occur as multi-subunit complexes. Some of these complexes contain multiple copies of the same subunit. TNF α and TNF β (collectively referred to hereafter as TNF) are homotrimers formed by three identical subunits (1-4). Other ligands are composed of non-identical
30 subunits. For example, hCG is a heterodimer (5-7). Receptors may also occur or function as multi-chain complexes. For example, receptors for TNF transduce a signal after being aggregated to form dimers (8,9). Ligands to these receptors promote aggregation of two or three receptor chains, thereby
35 affording a mechanism of receptor activation. For example, TNF-mediated aggregation activates TNF receptors (10-12).

 The modulation of protein-protein interactions can be a useful mechanism for therapeutic intervention in various diseases and pathologies. Soluble binding proteins, that can

interact with ligands, can potentially sequester the ligand away from the receptor, thereby reducing the activation of that particular receptor pathway. Alternatively, sequestration of the ligand may delay its elimination or degradation, thereby increasing its duration of effect, and perhaps its apparent activity *in vivo*. In the case of TNF, soluble TNF receptors have been primarily associated with inhibition of TNF activity (13-17).

Soluble binding proteins may be useful for treating human diseases. For example, soluble TNF receptors have been shown to have efficacy in animal models of arthritis (18,19).

Since TNF has three binding sites for its receptor (10-12), and dimerization of the cell surface receptor is sufficient for bioactivity (8,9), it is likely that binding of a single soluble receptor to TNF will leave open the possibility that this 1:3 complex of soluble receptor:TNF (trimer) can still bind and activate a pair of cell surface TNF receptors. To achieve an inhibitory effect, it would be expected that two of the receptor binding sites on the TNF trimer must be occupied or blocked by the soluble binding protein. Alternatively, the binding protein could block proper orientation of TNF at the cell surface.

Generally speaking, the need was felt of synthesizing proteins that contain two receptor (or ligands) chains, as dimeric hybrid protein. See Wallach et al., U.S. patent 5,478,925.

The primary strategy employed for generating dimeric or multimeric hybrid proteins, containing binding domains from extracellular receptors, has been to fuse these proteins to the constant regions of an antibody heavy chain.

This strategy led, for example, to the construction of CD4 immunoadhesins (20). These are hybrid molecules consisting of the first two (or all four) immunoglobulin-like domains of CD4 fused to the constant region of antibody heavy and light chains. This strategy for creating hybrid molecules was adapted to the receptors for TNF (10,16,21) and led to the generation of constructs with higher *in vitro* activity than the monomeric soluble binding proteins.

It is widely held that the higher *in vitro* potency of the dimeric fusion proteins should translate into higher *in vivo* activity. One study does support this, revealing an at least 50-fold higher activity for a p75(TBP2)-Ig fusion protein in protecting mice from the consequences of intravenous LPS injection (16).

However, despite the widespread utilization of immunoglobulin fusion proteins, this strategy has several drawbacks. One is that certain immunoglobulin Fc domains participate in effector functions of the immune system. These functions may be undesirable in a particular therapeutic setting (22).

A second limitation pertains to the special cases where it is desirable to produce heteromeric fusion proteins, for example soluble analogs of the heteromeric IL-6 or type I interferon receptors. Although there are numerous methods for producing bifunctional antibodies (e.g., by co-transfection or hybridoma fusions), the efficiency of synthesis is greatly compromised by the mixture of homodimers and heterodimers that typically results (23). Recently there have been several reports describing the use of leucine zipper motifs to guide assembly of heterodimers (24-26). This appears to be a promising approach for research purposes, but the non-native or intracellular sequences employed may not be suitable for chronic applications in the clinic due to antigenicity. The efficiency of assembly and stability post assembly may also be limitations.

On the other hand, in the particular case of TNF receptors, certain modifications to the p55 TNF receptor have been found to facilitate homodimerization and signaling in the absence of ligand (27,28). It has been found that a cytoplasmic region of the receptor, termed the "death domain," can act as a homodimerization motif (28,30). As an alternative to an immunoglobulin hybrid protein, fusion of the extracellular domain of the TNF receptor to its cytoplasmic death domain could conceivably result in a secreted protein which can dimerize in the absence of TNF. Such fusion proteins

have been disclosed and claimed in the International Patent Application WO 95/31544.

A third further strategy employed for generating dimers of soluble TNF receptors has been to chemically cross-link the monomeric proteins with polyethylene glycol (31).

SUMMARY OF THE INVENTION

An alternative for obtaining such dimeric proteins, offering some important advantages, is the one of the present invention and consists in using a natural heterodimeric scaffold corresponding to a circulating non-immunoglobulin protein with a long half-life. A preferred example is hCG, a protein that is secreted well, has good stability, and has a long half-life (32-33). Given hCG's prominent role as a marker of pregnancy, many reagents have been developed to quantitate and study the protein *in vitro* and *in vivo*. In addition, hCG has been extensively studied using mutagenesis, and it is known that small deletions to the protein, such as removal of five residues at the extreme carboxyl-terminus of the α subunit, can effectively eliminate its biological activity while preserving its capability to form heterodimer (34,35). Small insertions, of up to 30 amino acids, have been shown to be tolerated at the amino- and carboxyl-termini of the α subunit (36), while fusion of the α subunit to the carboxyl terminus of the β subunit also had little effect on heterodimer formation (37).

An analog of hCG in which an immunoglobulin Fc domain was fused to the C-terminus of hCG β subunit has also been reported; however, this construct was not secreted and no effort was made to combine it with an α subunit (38).

Therefore, the main object of the present invention is a hybrid protein comprising two coexpressed amino acid sequences forming a dimer, each comprising:

a) at least one amino acid sequence selected among a homomeric receptor, a chain of a heteromeric receptor, a ligand, and fragments thereof; and

b) a subunit of a heterodimeric proteinaceous hormone, or fragments thereof; in which (a) and (b) are bonded

directly or through a peptide linker, and in each couple the two subunits (b) are different and capable of aggregating forming a dimer complex.

5 According to the present invention, the linker may be enzymatically cleavable.

 Sequence (a) is preferably selected among: the extracellular domain of the TNF Receptor 1 (55 kDa, also called TBP1), the extracellular domain of the TNF Receptor 2
10 (75 kDa, also called TBP2), or fragments thereof still containing the ligand binding domain; the extracellular domains of the IL-6 receptors (also called gp80 and gp130); the extracellular domain of the IFN α/β receptor or IFN γ receptor; a gonadotropin receptor or its extracellular
15 fragments; antibody light chains, or fragments thereof, optionally associated with the respective heavy chains; antibody heavy chains, or fragments thereof, optionally associated with the respective light chains; antibody Fab domains; or ligand proteins, such as cytokines, growth factors
20 or hormones other than gonadotropins, specific examples of which include IL-6, IFN- β , TPO, or fragments thereof.

 Sequence (b) is preferably selected among a hCG, FSH, LH, TSH, inhibin subunit, or fragments thereof.

 Modifications to the proteins, such as chemical or
25 protease cleavage of the protein backbone, or chemical or enzymatic modification of certain amino acid side chains, can be used to render the components of the hybrid protein of the invention inactive. This restriction of activity may also be accomplished through the use of recombinant DNA techniques to
30 alter the coding sequence for the hybrid protein in a way that results directly in the restriction of activity to one component, or that renders the protein more amenable to subsequent chemical or enzymatic modification.

 The above hybrid proteins will result in
35 monofunctional, bifunctional or multifunctional molecules, depending on the amino acid sequences (a) that are combined with (b). In each couple, (a) can be linked to the amino termini or to the carboxy termini of (b), or to both.

A monoclonal hybrid protein of the present invention can, for instance, comprise the extracellular domain of a gonadotropin receptor linked to one of the corresponding
5 receptor-binding gonadotropin subunits. According to such an embodiment, the hybrid protein of the invention can be a molecule in which, for example, the FSH receptor extracellular domain is linked to FSH to increase plasma half-life and improve biological activity.

10 This preparation can be employed to induce follicular maturation in assisted reproduction methods, such as ovulation induction or in vitro fertilisation, and to serve as a means to dramatically amplify the biological activity of the hormone essential for the success of the process, thus reducing the
15 requirement for both the hormone itself and the number of injections to achieve ovulation.

The FSH receptor and the production of the extracellular domain of the human FSH receptor have been described respectively in WO 92/16620 and WO 96/38575.

20 According to a particular embodiment, the extracellular domain of the FSH receptor (ECD) can be fused in frame with a peptide linker that contains the thrombin recognition/cleavage site (29) and represents a "tethered" arm. The peptide linker links the extracellular domain of FSH with a
25 FSH subunit. This will allow for removal of the extracellular domain of the FSH receptor by cleavage at the thrombin cleavage site as the molecule comes in contact with thrombin in the systemic circulation.

30 In another embodiment, instead of the thrombin cleavage site, an enzyme recognition site for an enzyme that is found in greatest abundance in the ovary is used. In this way, as the ECD-FSH molecule travels to the ovary, it will be exposed to enzymes found in the highest concentrations in that tissue and the ECD will be removed so that the FSH can interact
35 with the membrane bound receptor.

In yet another embodiment, instead of an enzyme recognition site, a flexible hinge region is cloned between ECD and FSH so that the ECD will not be enzymatically removed from the hormone. In this way, when the ECD-FSH molecule arrives at

the ovary, a competition will be established between the hinge-attached ECD and the ECD of the FSH receptor found on the ovarian cell membrane.

5 In a further preferred embodiment of the invention, the hybrid protein consists of the aggregation between a couple of aa sequences, one of which contains TBP1 (or the fragments from aa 20 to aa 161 or to aa 190) as (a) and the α subunit of hCG as (b), and the other contains always TBP1 (or the same
10 fragments as above) as (a) and the β subunit of hCG, or fragments thereof, as (b). According to this embodiment, depending on the particular sequence that is chosen as (b) (the entire β subunit of hCG, or fragments or modifications thereof), the resulting hybrid protein will have one activity
15 (only that of TBP1) or a combination of activities (that of TBP1 with that of hCG). In this latter case the hybrid protein can be used, for example, in the combined treatment of Kaposi's sarcoma and metabolic wasting in AIDS.

In a further embodiment of the invention, one or more
20 covalent bonds between the two subunits (b) are added to enhance the stability of the resulting hybrid protein. This can be done, e.g., by adding one or more non-native interchain disulfide bonds. The sites for these cross-links can be deduced from the known structures of the heterodimeric
25 hormones. For example, a suitable site in hCG could be to place cysteine residues at α subunit residue Lys45 and β subunit residue Glu21, replacing a salt bridge (non-covalent bond) with a disulfide bond (covalent bond). Another object of the present invention are PEGylated or other chemically
30 modified forms of the hybrid proteins.

A further object of the present invention is a DNA molecule comprising the DNA sequence coding for the above hybrid protein, as well as nucleotide sequences substantially the same. "Nucleotide sequences substantially the same"
35 includes all other nucleic acid sequences which, by virtue of the degeneracy of the genetic code, also code for the given amino acid sequence.

For the production of the hybrid protein of the invention, the DNA sequence (a) is obtained from existing

clones, as is (b). The DNA sequence coding for the desired sequence (a) is ligated with the DNA sequence coding for the desired sequence (b). Two of these fused products are inserted and ligated into a suitable plasmid or each into a different plasmid. Once formed, the expression vector, or the two expression vectors, is introduced into a suitable host cell, which then expresses the vector(s) to yield the hybrid protein of the invention as defined above.

The preferred method for preparing the hybrid of the invention is by way of PCR technology using oligonucleotides specific for the desired sequences to be copied from the clones encoding sequences (a) and (b).

Expression of any of the recombinant proteins of the invention as mentioned herein can be effected in eukaryotic cells (e.g., yeasts, insect or mammalian cells) or prokaryotic cells, using the appropriate expression vectors. Any method known in the art can be employed.

For example the DNA molecules coding for the proteins obtained by any of the above methods are inserted into appropriately constructed expression vectors by techniques well known in the art (see Sambrook et al, 1989). Double stranded cDNA is linked to plasmid vectors by homopolymeric tailing or by restriction linking involving the use of synthetic DNA linkers or blunt-ended ligation techniques: DNA ligases are used to ligate the DNA molecules and undesirable joining is avoided by treatment with alkaline phosphatase.

In order to be capable of expressing the desired protein, an expression vector should comprise also specific nucleotide sequences containing transcriptional and translational regulatory information linked to the DNA coding the desired protein in such a way as to permit gene expression and production of the protein. First in order for the gene to be transcribed, it must be preceded by a promoter recognizable by RNA polymerase, to which the polymerase binds and thus initiates the transcription process. There are a variety of such promoters in use, which work with different efficiencies (strong and weak promoters).

For eukaryotic hosts, different transcriptional and translational regulatory sequences may be employed, depending on the nature of the host. They may be derived from viral sources, such as adenovirus, bovine papilloma virus, Simian virus or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. Examples are the TK promoter of the Herpes virus, the SV40 early promoter, the yeast gal4 gene promoter, etc.

Transcriptional initiation regulatory signals may be selected which allow for repression and activation, so that expression of the genes can be modulated.

The DNA molecule comprising the nucleotide sequence coding for the hybrid protein of the invention is inserted into a vector(s), having the operably linked transcriptional and translational regulatory signals, which is capable of integrating the desired gene sequences into the host cell. The cells which have been stably transformed by the introduced DNA can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may also provide for phototrophy to a auxotrophic host, biocide resistance, e.g., antibiotics, or heavy metals such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of proteins of the invention.

Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Once the vector(s) or DNA sequence containing the construct(s) has been prepared for expression, the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means: transformation,

transfection, conjugation, protoplast fusion, electroporation, calcium phosphate-precipitation, direct microinjection, etc.

Host cells may be either prokaryotic or eukaryotic.

5 Preferred are eukaryotic hosts, e.g., mammalian cells, such as human, monkey, mouse, and Chinese hamster ovary (CHO) cells, because they provide post-translational modifications to protein molecules, including correct folding or glycosylation at correct sites. Also, yeast cells can carry out post-
10 translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene
15 products and secretes peptides bearing leader sequences (i.e., pre-peptides).

After the introduction of the vector(s), the host cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned
20 gene sequence(s) results in the production of the desired proteins.

Purification of the recombinant proteins is carried out by any one of the methods known for this purpose, i.e., any conventional procedure involving extraction, precipitation,
25 chromatography, electrophoresis, or the like. A further purification procedure that may be used in preference for purifying the protein of the invention is affinity chromatography using monoclonal antibodies which bind the target protein and which are produced and immobilized on a gel
30 matrix contained within a column. Impure preparations containing the recombinant protein are passed through the column. The protein will be bound to the column by the specific antibody while the impurities will pass through. After washing, the protein is eluted from the gel by a change
35 in pH or ionic strength.

The term "hybrid protein", as used herein, generically refers to a protein which contains two or more different proteins or fragments thereof.

As used herein, "fusion protein" refers to a hybrid protein, which consists of two or more proteins, or fragments thereof, linked together covalently.

5 The term "aggregation", as used herein, means the formation of strong specific non-covalent interactions between two polypeptide chains forming a complex, such as those existing between the α and β subunit of a heterodimeric hormone (such as FSH, LH, hCG or TSH).

10 The terms "ligand" or "ligand protein", as used herein, refer to a molecule, other than an antibody or an immunoglobulin, capable of being bound by the ligand-binding domain of a receptor; such molecule may occur in nature, or may be chemically modified or chemically synthesised.

15 The term "ligand-binding domain", as used herein, refers to a portion of the receptor that is involved in binding a ligand and is generally a portion or essentially all of the extracellular domain.

20 The term "receptor", as used herein, refers to a membrane protein, whose binding with the respective ligand triggers secondary cellular responses that result in the activation or inhibition of intracellular process.

25 In a further aspect, the present invention provides the use of the hybrid protein as a medicament. The medicament is preferably presented in the form of a pharmaceutical composition comprising the protein of the invention together with one or more pharmaceutically acceptable carriers and/or excipients. Such pharmaceutical compositions represent yet a further aspect of the invention.

30 BRIEF DESCRIPTION OF THE DRAWINGS

 The invention will be better understood by reference to the appended drawings, in which:

35 Figures 1(a) and 1(b) show the TBP(20-161)-hCG α and TBP(20-161)-hCG β constructs, respectively, and the corresponding sequences (SEQ ID NOS:1-4).

 Figures 2(a) and 2(b) show the TBP(20-190)-hCG α and TBP(20-190)-hCG β constructs, respectively, and the corresponding sequences (SEQ ID NOS:5-8).



Figure 3 is a schematic summary of the constructs of Figures 1 and 2 showing p55 TNFR1, TBP1 and TBP1 fusion constructs. The linker sequences shown on the last two lines are SEQ ID NO:9 (Ala-Gly-Ala-Ala-Pro-Gly) and SEQ ID NO:10 (Ala-Gly-Ala-Gly).

Figure 4 is a graph illustrating the dose dependent protective effect of CHO cell expressed TBP-hCG(20-190) on TNF α -induced cytotoxicity on BT-20 cells and various controls.

Figure 5 is a graph illustrating the dose dependent protective effect of COS cell expressed TBP-hCG(20-190) on TNF α -induced cytotoxicity on BT-20 cells and various controls.

Figure 6 is a graph illustrating the dose dependent protective effect of affinity purified CHO cell expressed TBP-hCG(20-161) on TNF α -induced cytotoxicity on BT-20 cells and various controls.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention.

EXAMPLES

Materials and Methods

Cell lines used in this study were obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, unless otherwise specified. The CHO-DUKX cell line was obtained from L. Chasin at Columbia University through D. Houseman at MIT (39). The CHO-DUKX cells, which lack a functional gene for dihydrofolate reductase, were routinely maintained in complete α -plus Modified Eagles Medium (α (+)MEM) supplemented with 10% fetal bovine serum (FBS). The COS-7 cells were routinely maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS. Unless specified otherwise, cells were split to maintain them in log phase of growth, and culture reagents were obtained from GIBCO (Grand Island, New York).

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REPLACED

1. Assembly of the genetic constructs encoding the hybrid proteins

The numbering assignments for the p55 TNF receptor are based on the cloning paper from Wallach (40), while the numbering assignments for the hCG subunits are based on the numbering assignments from the Fiddes cloning papers (41,42). The designation TBP, or TNF binding protein, refers to the extracellular domain portions of the TNF receptors capable of binding TNF. In these Examples, the DNA constructs will be named as TBP-hybrid proteins, with the partner and region of TBP indicated in the construct nomenclature. All of the TBP-hCG constructs contain the human growth hormone (hGH) signal peptide in place of the native p55 signal sequence. In addition, the hGH signal peptide has been placed so that it immediately precedes TBP residue Asp20, which is anticipated to make this the first residue in the mature, secreted protein. These modifications are not essential to the basic concept of using hCG as a partner of the hybrid protein.

The DNAs encoding the hybrid proteins were constructed using PCR methodology (43).

a. TBP1(20-161)-hCG

The initial TBP-hCG construct was engineered to contain the ligand binding domain from the extracellular region of the p55 TNF receptor (from Asp20 inclusive of residue Cys161) fused though a short linker to the hCG α and β subunits (starting at residues α Cys7 or β Pro7, respectively). This construct, hereafter referred to as TBP1(20-161)-hCG, is a heterodimer of two modified hCG subunits, TBP1(20-161)-hCG α and TBP1(20-161)-hCG β .

The oligodeoxynucleotide primers used for the TBP1(20-161)-hCG α construct were:

primer 1($\alpha\beta$) TTT TCT CGA GAT GGC TAC AGG TAA GCG
CCC (SEQ ID NO:11)

primer 2(α) ACC TGG GGC AGC ACC GGC ACA GGA GAC ACA
CTC GTT TTC (SEQ ID NO:12)

primer 3(α) TGT GCC GGT GCT GCC CCA GGT TGC CCA GAA
TGC ACG CTA CAG (SEQ ID NO:13)

primer 4(α) TTT TGG ATC CTT AAG ATT TGT GAT AAT AAC
 AAG TAC (SEQ ID NO:14)

5 These and all of the other primers described in these
Examples were synthesized on an Applied Biosystems Model 392
DNA synthesis machine (ABI, Foster City, California), using
phosphoramidite chemistry.

10 Since both of the TBP-hCG subunit constructs have the
same 5'-end (i.e., the 5'-end of the hGH/TBP construct), primer
1($\alpha\beta$) was used for both TBP-hCG subunit constructs. The
other primers used for the TBP1(20-161)-hCG β construct were:

 primer 2(β) CCG TGG ACC AGC ACC AGC ACA GGA GAC
 ACA CTC GTT TTC (SEQ ID NO:15)

15 primer 3(β) TGT GCT GGT GCT GGT CCA CGG TGC CGC
 CCC ATC AAT (SEQ ID NO:16)

 primer 4(β) TTT TGG ATC CTT ATT GTG GGA GGA TCG
 GGG TG (SEQ ID NO:17)

20 Primers 2(α) and 3(α) are reverse complements, and
cover both the 3'-end of the coding region for the p55
extracellular domain, and the 5'-end of the hCG α subunit.
Similarly, primers 2(β) and 3(β) are also reverse
complements, and cover both the 3'-end of the coding region for
the p55 extracellular domain, and the 5'-end of the hCG β
subunit.

25 Two PCR reactions were run for each of the two TBP-
hCG subunit constructs. The first used primers 1($\alpha\beta$) and 2
(α or β), and used as the template a plasmid encoding soluble
p55 residues 20-180 preceded by the hGH signal peptide (plasmid
pCMVhGHspcDNA.pA4). The second used primers 3 (α or β) and 4
30 (α or β), and used as the template either plasmid pSVL-hCG α
or pSVL-hCG β (44). The PCR was performed using Vent (TM)
polymerase from New England Biolabs (Beverly, Massachusetts) in
accordance with the manufacturer's recommendations, using for
each reaction 25 cycles and the following conditions:

35 100 μ g of template DNA
 1 μ g of each primer
 2U of Vent(TM) polymerase (New England Biolabs)
 denaturation at 99°C for 30 seconds
annealing at: 59°C for 30 seconds for primers 1($\alpha\beta$) and 2(α)

59°C for 30 seconds for primers 3(α) and 4(α)
57°C for 30 seconds for primers 1($\alpha\beta$) and 2(β)
63°C for 30 seconds for primers 3(β) and 4(β)
extension at 75°C for 75 seconds.

The PCR products were confirmed to be the expected size by electrophoresis in a 2% agarose gel and ethidium bromide staining. The fragments were then purified by passage over a Wizard column (Promega) in accordance with the column manufacturer's recommendations.

The final coding sequence for TBP1(20-161)-hCG α was assembled by fusion PCR using primer 1($\alpha\beta$) and primer 4(α), and using as template the purified products from the p55 and hCG α fragments obtained from the first PCR reactions. First the two templates, which due to the overlap between primers 2(α) and 3(α) could be denatured and annealed together, were passed through 10 cycles of PCR in the absence of any added primers. The conditions for these cycles were essentially the same as those used earlier, except that the annealing was done at 67°C and the extension was performed for 2 minutes. At the end of these 10 cycles, primers 1($\alpha\beta$) and 4(α) were added, and another 10 cycles were performed. The conditions for this final set of reactions was the same as used earlier, except that an annealing temperature of 59°C was used, and the extension was performed for 75 seconds.

Analysis of the products of this reaction by electrophoresis in a 1% agarose gel confirmed that the expected fragment of about 1100bp was obtained. The reaction was passed over a Wizard column to purify the fragment, which was then digested with XbaI and BamHI and re-purified in a 0.7% low-melting point agarose gel. The purified fragment was subcloned into plasmid pSVL (Pharmacia), which had first been digested with XbaI and BamHI and gel purified on a 0.8% low-melting point agarose gel. Following ligation with T4 ligase, the mixture was used to transform AG1 E. coli and then plated onto LB/ampicillin plates for overnight culture at 37°C. Plasmid DNAs from ampicillin-resistant colonies were analyzed by digestion with XhoI and BamHI to confirm the presence of the insert (which is excised in this digest). Six clones were

found to contain inserts, and one (clone 7) was selected for further advancement and designated pSVLTBPhCG α (containing TBP1(20-161)-hCG α). Dideoxy DNA sequencing (using SequenaseTM, U.S. Biochemicals, Cleveland, Ohio) of the insert in this vector confirmed that the construct was correct, and that no undesired changes had been introduced.

The final coding sequence for TBP1(20-161)-hCG β was assembled in a manner similar to that described for TBP1(20-161)-hCG α using fusion PCR and primers 1($\alpha\beta$) and 4(β), and using as template the purified products from the p55 and hCG β fragments obtained from the first PCR reactions. The resulting pSVL plasmid containing the insert of interest was designated pSVLTBPhCG β .

b. TBP(20-190)-hCG

A second set of TBP-hCG proteins was prepared by modification of the TBP(20-161)-hCG constructs to produce an analog containing TBP spanning from Asp20 to Thr190, in place of the 20-161 region in the initial analog. This was done by replacing the fragment between the BglII and XbaI sites in plasmid pSVLTBPhCG α with a PCR fragment containing the change. This PCR fragment was generated using fusion PCR. The primers were:

primer 1 TTT TAG ATC TCT TCT TGC ACA GTG GAC
(SEQ ID NO:18)

primer 2 TGT GGT GCC TGA GTC CTC AGT (SEQ ID NO:19)

primer 3 ACT GAG GAC TCA GGC ACC ACA GCC GGT GCT
GCC CCA GGT TG (SEQ ID NO:20)

primer 4 TTT TTC TAG AGA AGC AGC AGC AGC CCA TG
(SEQ ID NO:21)

Primers 1 and 2 were used to generate the sequence coding the additional p55 residues from 161-190. The PCR reaction was performed essentially as described earlier, using 1 μ g of each primer and pUC-p55 as template. Similarly, primers 3 and 4 were used to generate by PCR the linker between the 3'-end of the TBP-coding region, and the 5'-end of the hCG α subunit coding region, using as a template plasmid pSVLTBPhCG α . Products from these PCR reactions were confirmed

to be the correct size (about 296 bp and 121 bp respectively) by polyacrylamide gel electrophoresis (PAGE) on an 8% gel, and were then purified using a Wizard column. The design of primers 2 and 3 was such that they contained a region of overlap, so that the two PCR products (from primers 1 and 2, and from primers 3 and 4) could be annealed for fusion PCR with primers 1 and 4. Subsequent to the fusion reaction, the desired product of about 400 bp was confirmed and purified using a 1.5% agarose gel and a Wizard column. This DNA was then digested with BglII and XbaI, and ligated with BglII/XbaI-digested pSVLTBPhCG α . The presence of an insert in plasmids isolated from transformed AG1 *E. coli* was confirmed by digestion with BglII and XbaI. The new construct was designated pSVLTBP(20-190)-hCG α .

Similarly, plasmid pSVLTBPhCG β was modified by substitution of the BglII-XcmI fragment. However, this was done by subcloning of a single PCR product, rather than with a fusion PCR product. Primers 1 and 2b (see below) were used with pUC-p55 as the template.

primer 2b TTT TCC ACA GCC AGG GTG GCA TTG ATG GGG
 CGG CAC CGT GGA CCA GCA CCA GCT GTG GTG
 CCT GAG TCC TCA GTG (SEQ ID NO:22)

The resulting PCR product (about 337bp) was confirmed and purified as described above, digested with BglII and XcmI, and then ligated into BglII/XbaI-digested pSVLTBPhCG β . The presence of an insert in plasmids isolated from transformed AG1 *E. coli* was confirmed by digestion with BglII and XcmI. The new construct was designated pSVLTBP(20-190)-hCG β .

The new constructs were subsequently confirmed by DNA sequencing.

In addition to producing these new pSVL-based plasmids, these constructs were also subcloned into other expression vectors likely to be more suitable for stable expression in CHO, particularly vector D α , previously described as plasmid CLH3AXSV2DHFR (45). This was accomplished by converting a BamHI site flanking the inserts in the pSVL-based vectors to an XhoI site, and then excising the insert with XhoI and cloning it into XhoI digested D α .

2. Transient and stable expression of the hybrid proteins

Transfections of COS-7 cells (ATCC CRL 1651, ref. 46) for transient expression of the TBP-hCG hybrid proteins were performed using electroporation (47). Exponentially growing COS-7 cells were removed by trypsinization, collected by gentle centrifugation (800 rpm, 4 minutes), washed with cold phosphate buffered saline (PBS), pH 7.3-7.4, and then repelleted by centrifugation. Cells were resuspended at a concentration of 5×10^6 cells per 400 μ l cold PBS and mixed with 10 μ g of plasmid DNA in a prechilled 2 mm gap electroporation cuvette. For cotransfections, 5 μ g of each plasmid were used. The cuvette and cells were chilled on ice for a further 10 minutes, and then subjected to electroporation using a BTX Model 600 instrument and conditions of 125 V, 950 μ F and R=8. Afterward the cells were set to cool on ice for 10 minutes, transferred to a 15 ml conical tube containing 9.5 ml complete medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine) at room temperature, and left at room temperature for 5 minutes. After gentle mixing in the 15 ml tube, the entire contents was seeded onto two P100 plates and placed into a 37°C, 5% CO₂ incubator. After 18 hours the media was changed, and in some cases the new media contained only 1% or 0% FBS. After another 72 hours, the conditioned media was harvested, centrifuged to remove cells, and then stored frozen at -70°C.

Transfections of CHO-DUKX (CHO) cells for transient or stable expression were performed using calcium phosphate precipitation of DNA. Twenty-four hours prior to the transfection, exponentially growing CHO cells were plated onto 100 mm culture plates at a density of 7.5×10^5 cells per plate. On the day of the transfection, 10 μ g of plasmid DNA was brought to 0.5 ml in transfection buffer (see below), 31 μ l of 2 M CaCl₂ were added, the DNA-CaCl₂ solution was mixed by vortexing, and left to stand at room temperature for 45 minutes. After this the media was aspirated from the plates, the DNA was added to the cells using a sterile plastic pipette, and the cells were left at room temperature for 20 minutes. At

the end of this period, 5 ml of complete $\alpha(+)$ MEM containing 10% FBS was added to the plates, which were incubated at 37°C for 4-6 hours. The media was then aspirated off the plates, and the cells were subjected to a glycerol shock by incubating them with a solution of 15% glycerol in transfection buffer at 37°C for 3.5 minutes. After removal of the glycerol solution, the cells were washed twice with PBS, refed with 10 ml complete $\alpha(+)$ MEM, 10% FBS, and returned to the 37°C incubator. For stable transfections, after 48 hours the cells were split 1:10 and fed with selection medium (complete α -minus MEM (lacking nucleosides), 10% dialyzed FBS, and 0.02 μ M methotrexate). Non-transfected (non-resistant) cells were typically eliminated in 3-4 weeks, leaving a population of transfected, methotrexate-resistant cells.

3. Quantitation of expression

Secretion of the hybrid proteins by transfected cells was assessed using a commercial assay kit for soluble p55 (R&D Systems; Minneapolis, Minnesota) in accordance with the manufacturer's instructions. This assay also provides an estimate of the hybrid protein levels in conditioned and processed media, which served as the basis for selecting doses to be used in the bioassay.

4. Assessment of heterodimer formation

To assess the ability of the TBP-hCG subunit fusions to combine and form heterodimers, a sandwich immunoassay using antibodies to the hCG subunits was performed. In this assay, a monoclonal antibody to the hCG β subunit is coated onto microtiter plates and used for analyte capture. The primary detection antibody is a goat polyclonal raised against the human TSH α subunit (#082422G - Biodesign International; Kennebunkport, Maine), which is in turn detected using a horse radish peroxidase conjugated rabbit anti-goat polyclonal antibody (Cappel; Durham, North Carolina).

Several different anti-hCG β subunit antibodies were used in this work, all of which show no detectable cross-reactivity with the free α subunit. One of these antibodies (3/6) is used in the commercially available MAIAclone hCG assay kit (Biodata; Rome, Italy).

High-protein binding microtiter plates (Costar #3590) were coated with capture antibody by incubation (2 hours at 37°C) with 100 μ l/well of a 5 μ g/ml solution of antibody in coating buffer (PBS, pH 7.4, 0.1 mM Ca^{++} , 0.1 mM Mg^{++}). After washing once with wash solution (PBS, pH 7.4 + 0.1% Tween 20) the plate is blocked by completely filling the wells (~400 μ l/well) with blocking solution (3% bovine serum albumin (BSA; fraction V - A-4503 Sigma) in PBS, pH 7.4) and incubating for one hour at 37°C or overnight at 4°C. The plate is then washed twice with wash solution, and the reference and experimental samples, diluted in diluent (5 mg/ml BSA in PBS, pH 7.4) to yield a 100 μ l volume, are added. After incubating the samples and the plate for two hours at 37°C, the plate is again twice washed with wash solution. The primary detection antibody, diluted 1:5000 in diluent, is added (100 μ l/well) and incubated for one hour at 37°C. The secondary detection antibody (HRP conjugated rabbit anti-goat Ig), diluted 1:5000 in diluent, is added (100 μ l/well) and after incubation for one hour at 37°C, the plate is washed three times with wash solution. One hundred μ l of TMB substrate solution (Kirkegaard and Perry Laboratories) is added, the plate is incubated 20 minutes in the dark at room temperature, and then the enzymatic reaction is stopped by addition of 50 μ l/well 0.3M H_2SO_4 . The plate is then analyzed using a microtiter plate reader set for a wavelength of 450 nm.

5. Partial purification

To better quantitate the activities of these hybrid proteins, TBP-hCG hybrid proteins were partially purified by immunoaffinity chromatography. The antibody used was a monoclonal commercially available from R&D Systems (MAB #225). The column was CNBr-activated sepharose, charged with the antibody by following the manufacturer's (Pharmacia) instructions.

Conditioned media was collected from confluent T-175 flasks of each line using daily harvests of 50 ml SFMII media (GIBCO), five harvests for each line. The collections were subjected to centrifugation (1000 RPM) to remove cellular debris. The material was then assayed for TBP content using

the commercial immunoassay and concentrated (Centricon units by Amicon; Beverly, Massachusetts) so that the apparent TBP concentration was about 50 ng/ml.

5 Ten ml of the concentrated TBP-hCG (sample #18873) was brought to approximately 1 M NaCl by addition of NaCl and adjustment of the solution to a conductivity of approximately 85 mS/cm. This was passed through a 0.5 ml anti-TBP immunoaffinity column. The flow-through was collected and run
10 through the column a second time. After this the column was washed with 1 M NaCl in PBS. The bound TBP(20-161)-hCG was collected after elution with 50 mM citric acid (pH 2.5). The eluate (approximately 7 ml) was concentrated by filtration using Amicon Centricon-10's in accordance with the
15 manufacturer's (Amicon) instructions, to a volume of approximately 200 μ l. Approximately 800 μ l of PBS was added to bring the sample volume to 1 ml, which was stored at 4°C until tested by bioassay.

6. Assessment of anti-TNF activity

20 Numerous *in vitro* TNF-induced cytotoxicity assays have been described for evaluating analogs of soluble TNF receptors. We utilized an assay employing a human breast carcinoma cell line, BT-20 cells (ATCC HTB 19). The use of
25 these cells as the basis for a TNF bioassay has been described previously (48). These cells are cultured at 37°C in RPMI 1640 media supplemented with 10% heat-inactivated FBS. The cells were grown to a maximum 80-90% confluence, which entailed
splitting every 3-4 days with a seeding density of about 3×10^6 cells per T175cm² flask.

30 The BT-20 assay uses the inclusion of a cellular stain, crystal violet, as a detection method to assess survival of cells after treatment with TNF. Dead cells are unable to take up and retain the dye.

35 In brief, the protocol used for the assay of anti-TNF activity is the following. Recombinant human TNF α (R&D Systems) and the experimental samples are constituted in media (RPMI 1640 with 5% heat-inactivated FBS) and added to the wells of 96-well culture plates. The cells are then plated into these wells at a density of 1×10^5 cells/well. The quantity of

TNF α added was determined earlier in titration studies, and represents a dose at which about 50% of the cells are killed.

After addition of the samples, the cells are cultured for 48 hours at 39°C, after which the proportion of live cells is determined using crystal violet staining and a microtiter plate reader (570 nm).

RESULTS

1. Constructs under study

The designs of the hybrid proteins studied are briefly summarized below; two control proteins, a monomeric soluble p55 (r-hTBP-1) and a dimeric TBP-immunoglobulin fusion protein (TBP-IgG3) (prepared essentially as described in (10)), were studied for comparative purposes.

<u>Construct</u>	<u>TBP N-term</u>	<u>TBP C-term</u>	<u>Fusion partner</u>
r-hTBP-1	mix of 9 and 20	180	none
TBP-IgG3	mix of 9 and 20	190	IgG3 heavy chain constant region
TBP(20-161)-hCG	20	161	hCG α and hCG β (heterodimer)
TBP(20-190)-hCG	20	190	hCG α and hCG β (heterodimer)

The sequences of the DNAs encoding, TBP(20-190)-hCG and TBP(20-161)-hCG are provided in Figures 1 and 2, respectively. A schematic summary of the constructs is provided in Figure 3.

2. Secretion of TBP-hCG proteins

All of the constructs tested were found to be produced and secreted into culture media by transfected mammalian cells. Data illustrating this are shown in Tables 1 and 2.

3. TBP-hCG(α/β) fusion proteins assemble into heterodimers

The combination of TBP-hCG α and TBP-hCG β was confirmed using the sandwich assay for the hCG heterodimer.



Only the combined transfection of α and β subunit fusions resulted in heterodimer detection (Table 3).

4. TBP-hCG hybrid proteins exhibit increased activity over TBP monomer

Hybrid proteins produced in either COS-7 or CHO cells were found to be potent inhibitors of TNF α in the BT-20 bioassay. Some of the samples tested are summarized in Table 4.

Negative controls (conditioned media from mock transfections) were included for the 1x media samples.

As illustrated in Figures 4-6 (points on y-axis), addition of TNF (2.5 ng/ml) results in a clear reduction in live cell number (as assessed by OD 570). In every case, active samples have as a maximal protective effect the restoration of cell viability to the level seen in the absence of added TNF (i.e., the control labeled "cells alone").

The positive controls, r-hTBP-1 and TBP-IgG3, are both protective, showing a clear dose-dependence and ED50s of approximately 100 ng/ml for the r-hTBP-1 (Figs. 4-6) and about 1.5 ng/ml for TBP-IgG3 (Fig. 4) respectively.

The TBP-hCG constructs from 1x media (CHO or COS) or from the immunopurification show dose-dependent protection, with approximate ED50s ranging from 2-11 ng/ml (Figs. 4-6).

The results from the *in vitro* bioassay are reported in Table 5. The data indicate that the hybrid proteins inhibit TNF cytotoxicity, and that they are substantially more potent than the TBP monomer. The negative controls were devoid of protective activity.

In addition to the possibility that dimerization of TBP may increase potency, it is also possible that the activity of the hybrid proteins are not related to dimeric interaction with TBP, but rather to steric inhibition due to the partner of the hybrid interfering with soluble TBP/TNF binding to cell-surface TNF receptors.

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference

This Sheet Has Been Replaced



- 23 -

THIS SHEET HAS BEEN
REPLACED

herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional method steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

TABLES

Table 1: COS-7 transient expression (TBP ELISA)	
Hybrid Protein	Concentration (pg/ml)
TBP1	66
TBP-hCG α (20-161)	5.1
TBP-hCG β (20-161)	0.5
TBP-hCG(20-161)	2.7
control	<0.25

Constructs were expressed using pSVL (Pharmacia)

Table 2: COS-7 transient expression (TBP ELISA)	
Hybrid protein	Concentration (ng/ml)
TBP1	131
TBP-hCG α (20-190)	81
TBP-hCG β (20-190)	9
TBP-hCG(20-190)	62
control	<1

Constructs were expressed using a mouse metallothionein promoter-containing vector - pD α

Table 3: COS-7 transient expression (hCG heterodimer assay)	
Hybrid Protein	Concentration (ng/ml)
TBP1	<0.2
TBP-hCG α (20-190)	<0.2
TBP-hCG β (20-190)	<0.2
TBP-hCG(20-190)	38
control	<0.2

Constructs were expressed using a mouse
metallothionein promoter-containing vector - pD α

Table 4: Samples tested for anti-TNF activity		
Construct	Cell source	Nature of sample
r-hTBP-1	CHO	purified
TBP-IgG3	CHO	1x conditioned media
TBP(20-161)-hCG	CHO	immunopurified (anti-TBP)
TBP(20-190)-hCG	CHO	1x conditioned media
TBP(20-190)-hCG	COS	1x conditioned media

Table 5 :Preliminary Assessment of the hybrid proteins in TNF Cytotoxicity Assay		
<i>Construct</i>	<i>Fusion partner</i>	<i>Anti-TNF activity (ED50) in BT-20 bioassay[*]</i>
r-hTBP-1	none	100 ng/ml
TBP-IgG3	IgG3 heavy chain constant region	1.5 ng/ml
TBP(20-161)-hCG	hCG α and hCG β (heterodimer)	2 ng/ml
TBP(20-190)-hCG	hCG α and hCG β (heterodimer)	8-11 ng/ml

^{**}The quantitation of material for dosing and estimation of ED50 was made using the TBP ELISA.

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1049 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: cDNA
 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 278..1047

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Ile Cys Cys Thr Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys	
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 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg
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Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile
 85 90 95

Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg Lys Asn
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Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys
 115 120 125

Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln
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Cys Val Ser Cys Ala Gly Ala Ala Pro Gly Cys Pro Glu Cys Thr Leu
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Gln Glu Asn Pro Phe Phe Ser Gln Pro Gly Ala Pro Ile Leu Gln Cys
 180 185 190

Met Gly Cys Cys Phe Ser Arg Ala Tyr Pro Thr Pro Leu Arg Ser Lys
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Lys Thr Met Leu Val Gln Lys Asn Val Thr Ser Glu Ser Thr Cys Cys
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- (A) LENGTH: 1202 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 279..1199

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTCGAGATGG CTACAGGTAA GCGCCCCTAA AATCCCTTTG GGCACAATGT GTCCTGAGGG 60

GAGAGGTAGC GACCTGTAGA TGGGACGGGG GCACTAACCC TGAGGTTTGG GGCTTCTGAA 120

TGTGAGTATC GCCATGTAAG CCCAGTATTT GGCCAATGTC AGAAAGCTCC TGGTCCCTGG 180

AGGGATGGAG AGAGAAAAAC AAACAGCTCC TGGAGCAGGG AGAGTGCTGG CCTCTTGCTC 240

TCCGGCTCCC TCTGTTGCCC TGTGGTTTCT CCCCAGGC TCC CGG ACG TCC CTG 293
 Ser Arg Thr Ser Leu
 260

CTC CTG GCT TTT GGC CTG CTC TGC CTG CCC TGG CTT CAA GAG GGC AGT 341
 Leu Leu Ala Phe Gly Leu Leu Cys Leu Pro Trp Leu Gln Glu Gly Ser
 265 270 275

GCC GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT 389
 Ala Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn
 280 285 290

TCG ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC 437
 Ser Ile Cys Cys Thr Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp
 295 300 305

TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC 485
 Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly
 310 315 320 325

TCT TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC 533
 Ser Phe Thr Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser
 330 335 340

AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG 581
 Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val
 345 350 355

GAC Asp	CGG Arg	GAC Asp	ACC Thr	GTG Val	TGT Cys	GGC Gly	TGC Cys	AGG Arg	AAG Lys	AAC Asn	CAG Gln	TAC Tyr	CGG Arg	CAT His	TAT Tyr	629
360 365 370																
TGG Trp	AGT Ser	GAA Glu	AAC Asn	CTT Leu	TTC Phe	CAG Gln	TGC Cys	TTC Phe	AAT Asn	TGC Cys	AGC Ser	CTC Leu	TGC Cys	CTC Leu	AAT Asn	677
375 380 385																
GGG Gly	ACC Thr	GTG Val	CAC His	CTC Leu	TCC Ser	TGC Cys	CAG Gln	GAG Glu	AAA Lys	CAG Gln	AAC Asn	ACC Thr	GTG Val	TGC Cys	ACC Thr	725
390 395 400 405																
TGC Cys	CAT His	GCA Ala	GGT Gly	TTC Phe	TTT Phe	CTA Leu	AGA Arg	GAA Glu	AAC Asn	GAG Glu	TGT Cys	GTC Val	TCC Ser	TGT Cys	GCT Ala	773
410 415 420																
GGT Gly	GCT Ala	GGT Gly	CCA Pro	CGG Arg	TGC Cys	CGC Arg	CCC Pro	ATC Ile	AAT Asn	GCC Ala	ACC Thr	CTG Leu	GCT Ala	GTG Val	GAG Glu	821
425 430 435																
AAG Lys	GAG Glu	GGC Gly	TGC Cys	CCC Pro	GTG Val	TGC Cys	ATC Ile	ACC Thr	GTC Val	AAC Asn	ACC Thr	ACC Thr	ATC Ile	TGT Cys	GCC Ala	869
440 445 450																
GGC Gly	TAC Tyr	TGC Cys	CCC Pro	ACC Thr	ATG Met	ACC Thr	CGC Arg	GTG Val	CTG Leu	CAG Gln	GGG Gly	GTC Val	CTC Leu	CCC Pro	GCC Ala	917
455 460 465																
CTG Leu	CCT Pro	CAG Gln	GTG Val	GTG Val	TGC Cys	AAC Asn	TAC Tyr	CGC Arg	GAT Asp	GTG Val	CGC Arg	TTC Phe	GAG Glu	TCC Ser	ATC Ile	965
470 475 480 485																
CGG Arg	CTC Leu	CCT Pro	GGC Gly	TGC Cys	CCG Pro	CGC Arg	GGC Gly	GTG Val	AAC Asn	CCC Pro	GTG Val	GTC Val	TCC Ser	TAC Tyr	GCT Ala	1013
490 495 500																
GTG Val	GCT Ala	CTC Leu	AGC Ser	TGT Cys	CAA Gln	TGT Cys	GCA Ala	CTC Leu	TGC Cys	CGC Arg	CGC Arg	AGC Ser	ACC Thr	ACT Thr	GAC Asp	1061
505 510 515																
TGC Cys	GGG Gly	GGT Gly	CCC Pro	AAG Lys	GAC Asp	CAC His	CCC Pro	TTG Leu	ACC Thr	TGT Cys	GAT Asp	GAC Asp	CCC Pro	CGC Arg	TTC Phe	1109
520 525 530																
CAG Gln	GAC Asp	TCC Ser	TCT Ser	TCC Ser	TCA Ser	AAG Lys	GCC Ala	CCT Pro	CCC Pro	CCC Pro	AGC Ser	CTT Leu	CCA Pro	AGC Ser	CCA Pro	1157
535 540 545																
TCC Ser	CGA Arg	CTC Leu	CCG Pro	GGG Gly	CCC Pro	TCG Ser	GAC Asp	ACC Thr	CCG Pro	ATC Ile	CTC Leu	CCA Pro	CAA Gln	TAA		1202
550 555 560																

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 307 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ser	Arg	Thr	Ser	Leu	Leu	Leu	Ala	Phe	Gly	Leu	Leu	Cys	Leu	Pro	Trp
1				5					10					15	

Leu Gln Glu Gly Ser Ala Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile
 20 25 30
 His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys Gly Thr
 35 40 45
 Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg
 50 55 60
 Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu Arg His
 65 70 75 80
 Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile
 85 90 95
 Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg Lys Asn
 100 105 110
 Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys
 115 120 125
 Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln
 130 135 140
 Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu
 145 150 155 160
 Cys Val Ser Cys Ala Gly Ala Gly Pro Arg Cys Arg Pro Ile Asn Ala
 165 170 175
 Thr Leu Ala Val Glu Lys Glu Gly Cys Pro Val Cys Ile Thr Val Asn
 180 185 190
 Thr Thr Ile Cys Ala Gly Tyr Cys Pro Thr Met Thr Arg Val Leu Gln
 195 200 205
 Gly Val Leu Pro Ala Leu Pro Gln Val Val Cys Asn Tyr Arg Asp Val
 210 215 220
 Arg Phe Glu Ser Ile Arg Leu Pro Gly Cys Pro Arg Gly Val Asn Pro
 225 230 235 240
 Val Val Ser Tyr Ala Val Ala Leu Ser Cys Gln Cys Ala Leu Cys Arg
 245 250 255
 Arg Ser Thr Thr Asp Cys Gly Gly Pro Lys Asp His Pro Leu Thr Cys
 260 265 270
 Asp Asp Pro Arg Phe Gln Asp Ser Ser Ser Ser Lys Ala Pro Pro Pro
 275 280 285
 Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile
 290 295 300
 Leu Pro Gln
 305

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1147 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 278..1132

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCGAGATGGC TACAGGTAAG CGCCCCTAAA ATCCCTTTGG GCACAATGTG TCCTGAGGGG	60
AGAGGCAGCG ACCTGTAGAT GGGACGGGGG CACTAACCCCT CAGGTTTGGG GCTTTTGAAT	120
GTGAGTATGG CCATGTAAGC CCAGTATTTG CCCAATCTCA GAAAGCTCCT GGTCCCTGGA	180
GGGATGGAGA GAGAAAAACA AACAGCTCCT GGAGCAGGGA CACTCCTGGC CTCTTGCTCT	240
GCGGCTCCGT GTGTTGCCCT GTGGTTTCTC CCCACGC TCC CGG ACG TCC CTG CTC	295
Ser Arg Thr Ser Leu Leu	310
CTG GCT TTT GGC CTG CTC TGC CTG CCC TGG CTT CAA GAG GGC AGT GCC	343
Leu Ala Phe Gly Leu Leu Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala	315 320 325
GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG	391
Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser	330 335 340 345
ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT	439
Ile Cys Cys Thr Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys	350 355 360
CCA GGC CCG GGG CAG GAT ACC GAC TGC AGG GAG TGT GAG AGC GGC TCC	487
Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser	365 370 375
TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA	535
Phe Thr Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys	380 385 390
TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC	583
Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp	395 400 405
CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG	631
Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp	410 415 420 425
AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC ACC CTC TGC CTC AAT GGG	679
Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys Thr Leu Cys Leu Asn Gly	430 435 440
ACC GTG CAC CTC TCC TGT CAG GAG AAA CAG AAC ACC GTC TGC ACC TGC	727
Thr Val His Leu Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys	445 450 455
CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC TCC TGT AGT AAC	775
His Ala Gly Phe Phe Leu Arg Glu Asn Glu Cys Val Ser Cys Ser Asn	460 465 470
TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG TCC CTA CCC CAG ATT GAG	823
Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Ser Leu Pro Gln Ile Glu	475 480 485
AAT GTT AAG GGC ACT GAG GAC TCA GGC ACC ACA GCC GGT GCT GCC CCA	871
Asn Val Lys Gly Thr Glu Asp Ser Gly Thr Thr Ala Gly Ala Ala Pro	490 495 500 505

GGT TGC CCA GAA TGC ACG CTA CAG GAA AAC CCA TTC TTC TCC CAG CCG	919
Gly Cys Pro Glu Cys Thr Leu Gln Glu Asn Pro Phe Phe Ser Gln Pro	
510 515 520	
GGT GCC CCA ATA CTT CAG TGC ATG GGC TGC TGC TTC TCT AGA GCA TAT	967
Gly Ala Pro Ile Leu Gln Cys Met Gly Cys Cys Phe Ser Arg Ala Tyr	
525 530 535	
CCC ACT CCA CTA AGG TCC AAG AAG ACG ATG TTG GTC CAA AAG AAC GTC	1015
Pro Thr Pro Leu Arg Ser Lys Lys Thr Met Leu Val Gln Lys Asn Val	
540 545 550	
ACC TCA GAG TCC ACT TGC TGT GTA GCT AAA TCA TAT AAC AGG GTC ACA	1063
Thr Ser Glu Ser Thr Cys Cys Val Ala Lys Ser Tyr Asn Arg Val Thr	
555 560 565	
GTA ATG GGG GGT TTC AAA GTG GAG AAC CAC ACG GCG TGC CAC TGC AGT	1111
Val Met Gly Gly Phe Lys Val Glu Asn His Thr Ala Cys His Cys Ser	
570 575 580 585	
ACT TGT TAT TAT CAC AAA TCT TAAGGATCCC TCGAG	1147
Thr Cys Tyr Tyr His Lys Ser	
590	

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 285 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser	Arg	Thr	Ser	Leu	Leu	Leu	Ala	Phe	Gly	Leu	Leu	Cys	Leu	Pro	Trp
1				5					10					15	
Leu	Gln	Glu	Gly	Ser	Ala	Asp	Ser	Val	Cys	Pro	Gln	Gly	Lys	Tyr	Ile
			20					25					30		
His	Pro	Gln	Asn	Asn	Ser	Ile	Cys	Cys	Thr	Lys	Cys	His	Lys	Gly	Thr
		35					40					45			
Tyr	Leu	Tyr	Asn	Asp	Cys	Pro	Gly	Pro	Gly	Gln	Asp	Thr	Asp	Cys	Arg
	50				55						60				
Glu	Cys	Glu	Ser	Gly	Ser	Phe	Thr	Ala	Ser	Glu	Asn	His	Leu	Arg	His
65					70					75					80
Cys	Leu	Ser	Cys	Ser	Lys	Cys	Arg	Lys	Glu	Met	Gly	Gln	Val	Glu	Ile
			85						90					95	
Ser	Ser	Cys	Thr	Val	Asp	Arg	Asp	Thr	Val	Cys	Gly	Cys	Arg	Lys	Asn
			100					105					110		
Gln	Tyr	Arg	His	Tyr	Trp	Ser	Glu	Asn	Leu	Phe	Gln	Cys	Phe	Asn	Cys
		115					120					125			
Thr	Leu	Cys	Leu	Asn	Gly	Thr	Val	His	Leu	Ser	Cys	Gln	Glu	Lys	Gln
	130				135						140				
Asn	Thr	Val	Cys	Thr	Cys	His	Ala	Gly	Phe	Phe	Leu	Arg	Glu	Asn	Glu
145					150				155						160
Cys	Val	Ser	Cys	Ser	Asn	Cys	Lys	Lys	Ser	Leu	Glu	Cys	Thr	Lys	Leu
				165					170					175	

Ser Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser Gly Thr
 180 185 190

Thr Ala Gly Ala Ala Pro Gly Cys Pro Glu Cys Thr Leu Gln Glu Asn
 195 200 205

Pro Phe Phe Ser Gln Pro Gly Ala Pro Ile Leu Gln Cys Met Gly Cys
 210 215 220

Cys Phe Ser Arg Ala Tyr Pro Thr Pro Leu Arg Ser Lys Lys Thr Met
 225 230 235 240

Leu Val Gln Lys Asn Val Thr Ser Glu Ser Thr Cys Cys Val Ala Lys
 245 250 255

Ser Tyr Asn Arg Val Thr Val Met Gly Gly Phe Lys Val Glu Asn His
 260 265 270

Thr Ala Cys His Cys Ser Thr Cys Tyr Tyr His Lys Ser
 275 280 285

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1301 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 279..1287

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTCGAGATGG CTACAGGTAA GCGCCCCTAA AATCCCTTTG GGCACAATGT GTCCTGAGGG 60

GAGAGGCAGC GACCTGTAGA TGGGACGGGG GCACTAACCC TCAGGTTTGG GGCTTCTGAA 120

TGTGAGTATC GCCATGTAAG CCCAGTATTT GGCCAATGTC AGAAAGCTCC TGGTCCCTGG 180

AGGGATGGAG AGAGAAAAAC AAACACCTCC TGGAGCAGGG AGAGTGCTGC CCTCTTGCTC 240

TCCGGCTCCC TCTGTTGCCC TCTGGTTTCT CCCCAGGC TCC CGG ACG TCC CTG 293

Ser Arg Thr Ser Leu
290

CTC CTG GCT TTT GGC CTG CTC TGC CTG CCC TGG CTT CAA GAG GGC AGT 341

Leu Leu Ala Phe Gly Leu Leu Cys Leu Pro Trp Leu Gln Glu Gly Ser
295 300 305

GCC GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT 389

Ala Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn
310 315 320

TCG ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC 437

Ser Ile Cys Cys Thr Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp
325 330 335

TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC 485

Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly
340 345 350

TCC Ser 355	TTC Phe	ACC Thr	GCT Ala	TCA Ser	GAA Glu 360	AAC Asn	CAC His	CTC Leu	AGA Arg	CAC His 365	TGC Cys	CTC Leu	AGC Ser	TGC Cys	TCC Ser 370	533
AAA Lys	TGC Cys	CGA Arg	AAG Lys	GAA Glu 375	ATG Met	GGT Gly	CAG Gln	GTG Val	GAG Glu 380	ATC Ile	TCT Ser	TCT Ser	TGC Cys	ACA Thr 385	GTG Val	581
GAC Asp	CGG Arg	GAC Asp	ACC Thr 390	GTG Val	TGT Cys	GGC Gly	TGC Cys	AGG Arg 395	AAG Lys	AAC Asn	CAG Gln	TAC Tyr	CGG Arg 400	CAT His	TAT Tyr	629
TGG Trp	AGT Ser	GAA Glu 405	AAC Asn	CTT Leu	TTC Phe	CAG Gln	TGC Cys 410	TTC Phe	AAT Asn	TGC Cys	AGC Ser	CTC Leu 415	TGC Cys	CTC Leu	AAT Asn	677
GGG Gly 420	ACC Thr	GTG Val	CAC His	CTC Leu	TCC Ser	TGC Cys 425	CAG Gln	GAG Glu	AAA Lys	CAG Gln	AAC Asn 430	ACC Thr	GTG Val	TGC Cys	ACC Thr	725
TGC Cys 435	CAT His	GCA Ala	GGT Gly	TTC Phe 440	TTT Phe	CTA Leu	AGA Arg	GAA Glu	AAC Asn	GAG Glu 445	TGT Cys	GTC Val	TCC Ser	TGT Cys	AGT Ser 450	773
AAC Asn	TGT Cys	AAG Lys	AAA Lys	AGC Ser 455	CTG Leu	GAG Glu	TGC Cys	ACG Thr	AAG Lys 460	TTG Leu	TGC Cys	CTA Leu	CCC Pro	CAG Gln 465	ATT Ile	821
GAG Glu	AAT Asn	GTT Val	AAG Lys 470	GGC Gly	ACT Thr	GAG Glu	GAC Asp	TCA Ser 475	GGC Gly	ACC Thr	ACA Thr	GCT Ala	GGT Gly 480	GCT Ala	GGT Gly	869
CCA Pro	CGG Arg	TGC Cys 485	CGC Arg	CCC Pro	ATC Ile	AAT Asn	GCC Ala 490	ACC Thr	CTG Leu	GCT Ala	GTG Val	GAG Glu 495	AAG Lys	GAG Glu	GGC Gly	917
TGC Cys 500	CCC Pro	GTG Val	TGC Cys	ATC Ile	ACC Thr	GTC Val 505	AAC Asn	ACC Thr	ACC Thr	ATC Ile	TGT Cys 510	GCC Ala	GGC Gly	TAC Tyr	TGC Cys	965
CCC Pro 515	ACC Thr	ATG Met	ACC Thr	CGC Arg	GTG Val 520	CTG Leu	CAG Gln	GGG Gly	GTC Val	CTG Leu 525	CCG Pro	GCC Ala	CTG Leu	CCT Pro	CAG Gln 530	1013
GTG Val	GTG Val	TGC Cys	AAC Asn	TAC Tyr 535	CGC Arg	GAT Asp	GTG Val	CGC Arg	TTC Phe 540	GAG Glu	TCC Ser	ATC Ile	CGG Arg	CTC Leu 545	CCT Pro	1061
GGC Gly	TGC Cys	CCG Pro	CGC Arg 550	GGC Gly	GTG Val	AAC Asn	CCC Pro	GTG Val 555	GTC Val	TCC Ser	TAC Tyr	GCC Ala	GTG Val 560	GCT Ala	CTC Leu	1109
AGC Ser	TGT Cys	CAA Gln 565	TGT Cys	GCA Ala	CTC Leu	TGC Cys	CGC Arg 570	CGC Arg	AGC Ser	ACC Thr	ACT Thr	GAC Asp 575	TGC Cys	GGG Gly	GGT Gly	1157
CCC Pro 580	AAG Lys	GAC Asp	CAC His	CCC Pro	TTG Leu	ACC Thr 585	TGT Cys	GAT Asp	GAC Asp	CCC Pro	CGC Arg 590	TTC Phe	CAG Gln	GAC Asp	TCC Ser	1205
TCT Ser 595	TCC Ser	TCA Ser	AAG Lys	GCC Ala 600	CCT Pro	CCC Pro	CCC Pro	AGC Ser	CTT Leu 605	CCA Pro	AGC Ser	CCA Pro	TCC Ser	CGA Arg	CTC Leu 610	1253
CCG Pro	GGG Gly	CCC Pro	TCG Ser	GAC Asp 615	ACC Thr	CCG Pro	ATC Ile	CTC Leu	CCA Pro 620	CAA Gln	T AAGGATCCCT	CGAG				1301

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 336 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu Cys Leu Pro Trp
 1           5           10           15
Leu Gln Glu Gly Ser Ala Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile
          20           25           30
His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys Gly Thr
          35           40           45
Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg
 50           55           60
Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu Arg His
 65           70           75           80
Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile
          85           90           95
Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg Lys Asn
          100          105          110
Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys
          115          120          125
Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln
          130          135          140
Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu
          145          150          155          160
Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu
          165          170          175
Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser Gly Thr
          180          185          190
Thr Ala Gly Ala Gly Pro Arg Cys Arg Pro Ile Asn Ala Thr Leu Ala
          195          200          205
Val Glu Lys Glu Gly Cys Pro Val Cys Ile Thr Val Asn Thr Thr Ile
          210          215          220
Cys Ala Gly Tyr Cys Pro Thr Met Thr Arg Val Leu Gln Gly Val Leu
          225          230          235          240
Pro Ala Leu Pro Gln Val Val Cys Asn Tyr Arg Asp Val Arg Phe Glu
          245          250          255
Ser Ile Arg Leu Pro Gly Cys Pro Arg Gly Val Asn Pro Val Val Ser
          260          265          270
Tyr Ala Val Ala Leu Ser Cys Gln Cys Ala Leu Cys Arg Arg Ser Thr
          275          280          285

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Thr	Asp	Cys	Gly	Gly	Pro	Lys	Asp	His	Pro	Leu	Thr	Cys	Asp	Asp	Pro
290						295					300				
Arg	Phe	Gln	Asp	Ser	Ser	Ser	Ser	Lys	Ala	Pro	Pro	Pro	Ser	Leu	Pro
305					310					315					320
Ser	Pro	Ser	Arg	Leu	Pro	Gly	Pro	Ser	Asp	Thr	Pro	Ile	Leu	Pro	Gln
				325					330					335	

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Gly Ala Ala Pro Gly
 1 5

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 4 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala Gly Ala Gly
 1

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TTTTCTCGAG ATGGCTACAG GTAAGCGCCC

30

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 39 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ACCTGGGGCA GCACCGGCAC AGGAGACACA CTCGTTTTC

39

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 42 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGTGCCGGTG CTGCCCCAGG TTGCCAGAA TGCACGCTAC AG

42

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTTGGATCC TTAAGATTG TGATAATAAC AAGTAC

36

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 39 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCGTGGACCA GCACCAGCAC AGGAGACACA CTCGTTTTTC

39

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGTGCTGGTG CTGGTCCACG GTGCCGCCCC ATCAAT

36

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TTTTGGATCC TTATTGTGGG AGGATCGGGG TG

32

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTTTAGATCT CTTCTTGCAC AGTGGAC

27

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TGTGGTGCCT GAGTCCTCAG T

21

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ACTGAGGACT CAGGCACCAC AGCCGGTGCT GCCCCAGGTT G

41

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TTTTTCTAGA GAAGCAGCAG CAGCCCATG

29

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 75 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TTTTCCACAG CCAGGGTGGC ATTGATGGGG CGGCACCGTG GACCAGCACC AGCTGTGGTG	60
CCTGAGTCCT CAGTG	75

**CLAIMS**

1. A hybrid protein comprising two coexpressed amino acid sequences forming a dimer, each comprising:

a) at least one amino acid sequence selected from the group consisting of a homomeric receptor, a chain of a heteromeric receptor, a ligand, and fragments thereof which retain the ligand-receptor binding capability; and

b) a subunit of a heterodimeric proteinaceous hormone, or fragments thereof which retain the ability of the subunit to form a heterodimer with other subunits thereof;

wherein sequences (a) and (b) are bonded directly or through a peptide linker, and in which the sequence (b) in each of said two coexpressed sequences are capable of aggregating to form a dimer complex.

2. A hybrid protein in accordance with claim 1, wherein said sequence (a) is selected from the group consisting of TBP1, TBP2 or fragments thereof still containing the ligand binding domain; the extracellular domain of the IFN α/β receptor or the IFN γ receptor; a gonadotropin receptor or extracellular fragments thereof; antibody light chains or fragments thereof, optionally associated with the respective heavy chains; antibody heavy chains or fragments thereof; antibody Fab domains; and IL-6, IFN- β , TPO or fragments thereof.

3. A hybrid protein in accordance with claim 1, wherein said sequence (b) is selected from the group consisting of subunits of hCG, FSH, LH, TSH or inhibin, and fragments thereof.

4. A hybrid protein in accordance with claim 1, wherein sequence (a) is linked to the amino terminus of sequence (b).



5. A hybrid protein in accordance with claim 1, wherein sequence (a) is linked to the carboxy terminus of sequence (b).

6. A hybrid protein in accordance with claim 1, wherein said two coexpressed amino acid sequences each include the sequence for TBP1 or the fragment thereof corresponding to amino acid residues 20-161 or 20-190 of TBP1, as sequence (a) and the respective α and β subunits of hCG or fragments thereof, as sequence (b).

7. A hybrid protein in accordance with claim 1, wherein said two coexpressed amino acid sequences each include the extracellular domain of a gonadotropin receptor as sequence (a) and the respective α and β subunits of a gonadotropin as sequence (b).

8. A hybrid protein in accordance with claim 7, wherein said sequence (a) is the FSH receptor extracellular domain and sequence (b) is a subunit of FSH.

9. A hybrid protein in accordance with claim 7, wherein said sequences (a) and (b) are linked with a peptide linker.

10. A hybrid protein in accordance with claim 9, wherein said peptide linker has an enzyme cleavage site.

11. A hybrid protein in accordance with claim 10, wherein said enzyme cleavage site is a thrombin cleavage site.

12. A hybrid protein in accordance with claim 10, wherein said enzyme cleavage site is recognized and cleaved by an enzyme which is found in the ovary.

13. A hybrid protein in accordance with claim 9, wherein said peptide linker serves as a flexible hinge.



14. A hybrid protein in accordance with claim 1, wherein one or more covalent bonds between the two subunits (b) are added.

15. A DNA molecule encoding a hybrid protein in accordance with claim 1.

16. An expression vector containing a DNA molecule in accordance with claim 15.

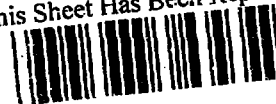
17. A host cell containing an expression vector in accordance with claim 16 and capable of expressing said hybrid protein.

18. A method for producing hybrid protein comprising culturing a host cell in accordance with claim 17 and recovering the hybrid protein expressed thereby.

19. A pharmaceutical composition comprising a hybrid protein in accordance with claim 1 and a pharmaceutically acceptable carrier and/or excipient.

20. A method for inducing follicular maturation, comprising administering a pharmaceutical composition comprising the hybrid protein of claim 8 to a subject in need thereof.

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Figure 1 (a)

TBP(20-161)-hCG α FUSION CONSTRUCT

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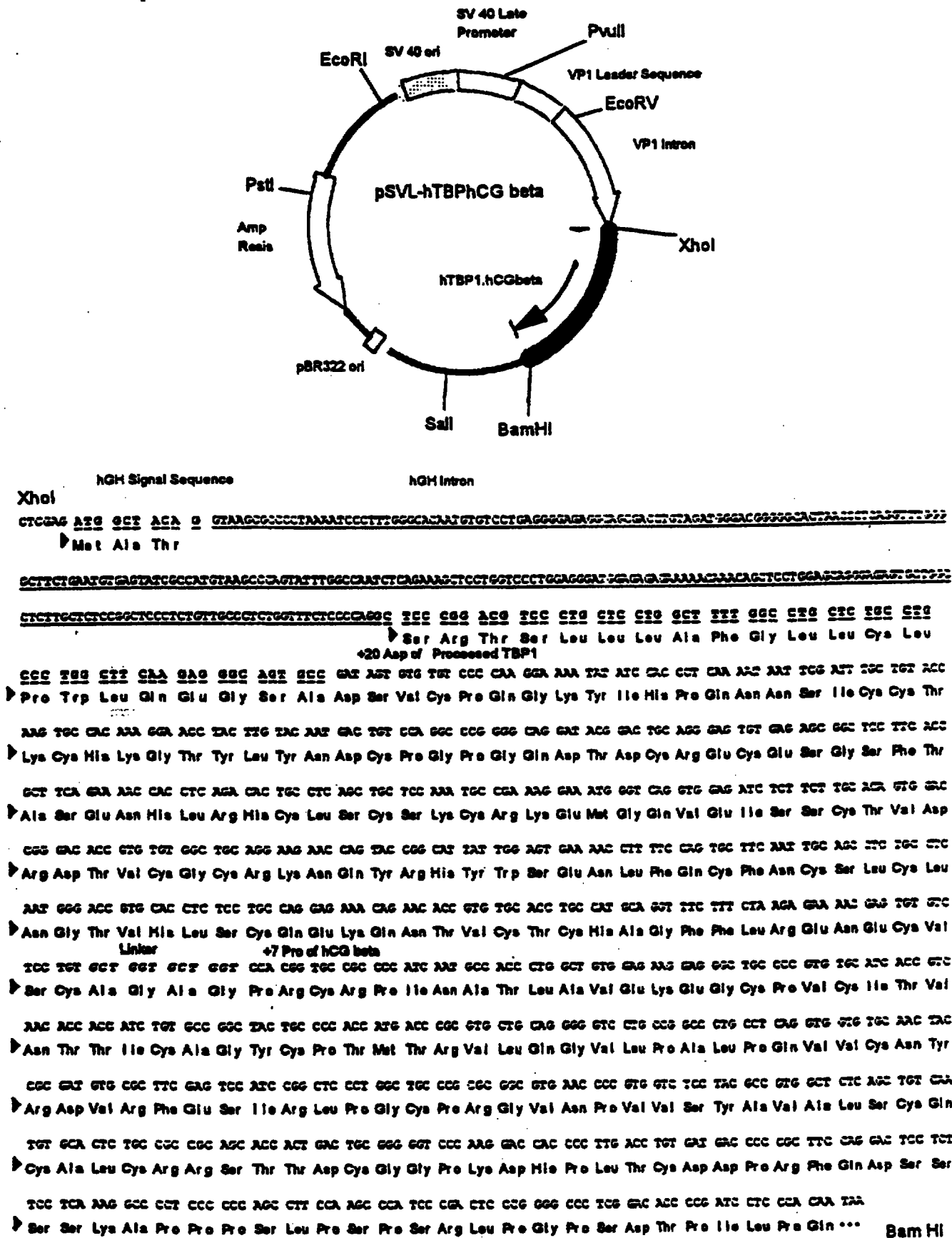


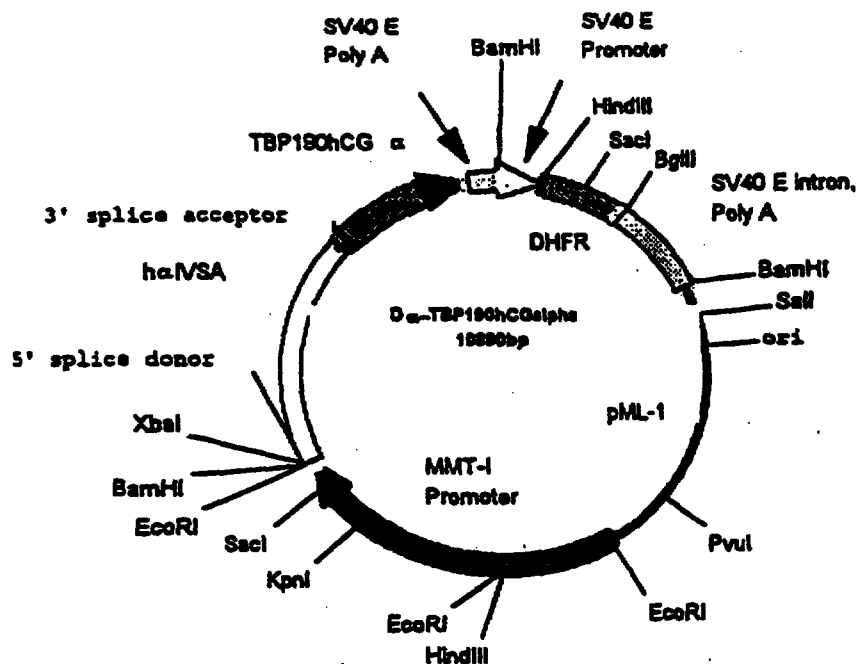
Figure 1 (b)

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TBP(20-161)-hCGβ FUSION CONSTRUCT

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XhoI hGH Signal Sequence hGH Intron

TGAG ATG GGT ACA C CTAAACCCGCCCTAAAATCGCCTTTCGGCACAAATGTGTCTTGAGTGGACAGGCACCGACCCTACATCGGACGGGTCACTAACCTCAAGTTCCGGTTCCTG
Met Ala Thr

GAATGTGACTATCGCATGTAAAGCCGAGTATTTCGCAATCTCAGAAAGCTCCTGCTCCCTGGAGGATGTGACAGCAAATAAACAACAGCTCTCGGAGCAGCGGAGAGTCTGGCCTCTCTTCTTCT

CGGCTCGGCTCTGTTTGGCCCTCTGGCTTTCGCGAGGG TGG CGG ACG TGG CTG CTC CTG GGT TTT GAG CTG GTC TGG CGG TGG GTT GTT
Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu Cys Leu Pro Trp Leu
+20 Asp of processed BMP1

GAA GAG GGC AGT GGC CAT AGT GTC TOT CCC CAA GGA AAA ZAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TOT ACC AAG TGC CAC AAA GGA
Gln Glu Gly Ser Ala Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys Gly

ACC TAC TTG TAC AAT GAC TOT CCA GCG CGG GGG CAG GAT AGC GAC TGC AGC GAG TOT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC
Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu

AGA CAG TGC CTC ACC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC CGG GAC ACC GTG TGT GGC TGC
Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys

AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC AAT GCG ACC GTG CAC CTC TCC TGC
Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys

CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TOT GTC TCC TOT AGT AAC TOT AAG AAA AGC CTG
Gln Glu Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu
Linker +7 Cys/hCG alpha

GAG TGC AGC AAG TTS TGC CTA CCC CAG ATT GAG AAT GTT AAG GGC ACT GAG GAC TCA GGC ACC ACA GGC GGT GGT GGC GGA GGT TGC CCA
Glu Cys Thr Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser Gly Thr Thr Ala Gly Ala Ala Pro Gly Cys Pro

GAA TGC AGC CTA CAG GAA AAC CCA TTC TTC TCC CAG CGG GGT GCC CCA ATA CTT CAG TGC ATG GGT TGC TGC TTC TCT AGA GCA TAT CCC AAT
Glu Cys Thr Leu Gln Glu Asn Pro Phe Phe Ser Gln Pro Gly Ala Pro Ile Leu Gln Cys Met Gly Cys Cys Phe Ser Arg Ala Tyr Pro Thr

CCA CTA AGC TCC AAG AAG ACC ATG TTS GTC CAA AAG AAC GTC ACC TCA CAG TCC ACT TGC TOT GTA GGT AAA TCA TAT AAC AGC GTC ACA GTA
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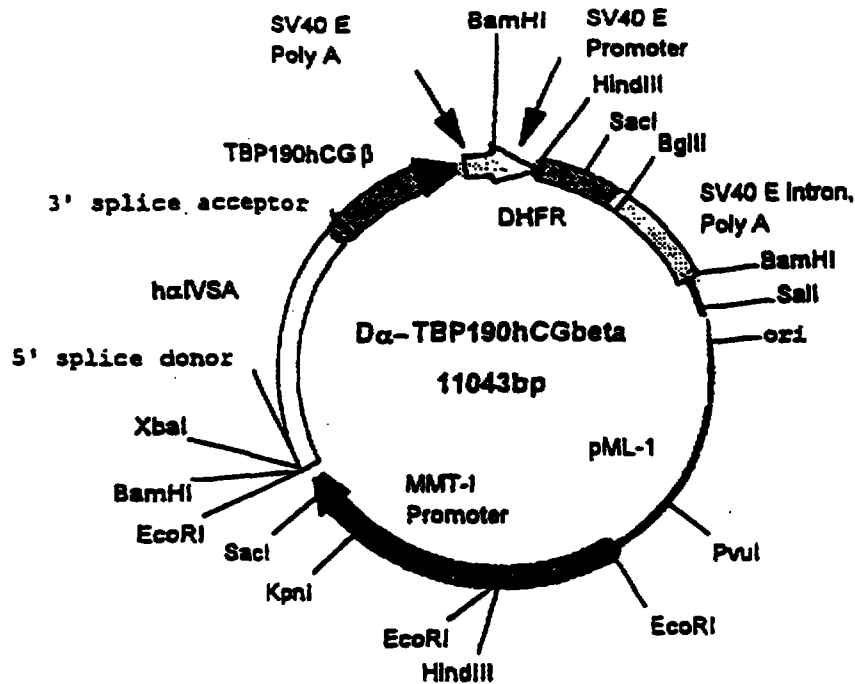
ATG GCG GGT TTC AAA GTG CAG AAC CAC AGC GCG TGC CAC TGC AAT ACT TOT TAT TAT CAC AAA TCT TAA GCATCCCTCGAG
Met Gly Gly Phe Lys Val Glu Asn His Thr Ala Cys His Cys Ser Thr Cys Tyr Tyr His Lys Ser ... BamHI XhoI

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Figure 2(a)
BP(20-190)-hCG α FUSION CONSTRUCT

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XbaI hGH Signal Sequence hGH Intron

CCTCGAG ATG GCG ACA G GTAAAGCCCGCTAAATCTCTTTGGGACAACTGTGTCTCTGAGGGGCGTGGACCCACCTTACGTGGGACGGTGGGCTTAAAGCTCAGCTTTGGG

Met Ala Thr

CCTCTGATGTTGATATGCGACGTAAAGCCCTGATTTGGGCAATCTCAGAAAGCTCTCTGGTCCCTGGAGGGATGCGAGAGCAAAACAGCTCTCTGATGAGGCGAGTCTGGG

CCTCTGCTCTCGGGCTCCCTCTGTTGGCCCTCTGGTTTGGCCAGGG TCC CCG ACC TCC CTC CTC CTC GCG TTT GCG CTC CTC TCC TCC CTC

Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu Cys Leu

+20 Asp of Processed TBP1

CGG TGG CTT CAA GAG GCG AGT GCG GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG AAT TCG TGT ACC

Pro Asp Leu Glu Glu Gly Ser Ala Asp Ser Val Cys Pro Glu Gly Lys Tyr Ile His Pro Glu Asn Asn Ser Ile Cys Cys Thr

AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GCG CCG GCG CAG GAT ACC GAC TGC ACC GAG TGT GAG ACC GCG TGC TTC ACC

Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Glu Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr

GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC ACC TGC TCC AAA TGC CCA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC

Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Glu Val Glu Ile Ser Ser Cys Thr Val Asp

CGG GAC ACC GTG TGT GCG TGC ACC AAG AAG CAG TAC CCG CAT TAT TCG AAT GAA AAC CTT TTC CAG TGC TTC AAT TGC ACC CTC TGC CTC

Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Glu Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Glu Cys Phe Asn Cys Ser Leu Cys Leu

ATG GCG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC

Asn Gly Thr Val His Leu Ser Cys Glu Glu Lys Glu Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu Cys Val

TCC TGT AGT AAC TGT AAG AAA ACC CTG GAG TGC ACC AAG TTG TGC CTA CCC CAG AAT GAG AAT GTT AAG GCG ACT GAG GAC TCA GCG ACC

Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys Leu Pro Glu Ile Glu Asn Val Lys Gly Thr Glu Asp Ser Gly Thr

Linker +7 Pro of beta

ACA GCT GGT GGT GGT CCA CCG TGC CCG CCC ATC AAT GCG ACC CTC GCT GTG GAG AAG GAG GCG TGC CCC GTG TGC ATC ACC GTC AAC

Thr Ala Gly Ala Gly Pro Arg Cys Arg Pro Ile Asn Ala Thr Leu Ala Val Glu Lys Glu Gly Cys Pro Val Cys Ile Thr Val Asn

ACC ACC ATC TGT GCG GAC TAC TGC CCC ACC ATG ACC CCG GTG CTC CAG GCG GTG CTC CCG GCG CTC CCT CAG GTG GTG TGC AAC TAC CCG

Thr Thr Ile Cys Ala Gly Tyr Cys Pro Thr Met Thr Arg Val Leu Glu Gly Val Leu Pro Ala Leu Pro Glu Val Val Cys Asn Tyr Arg

GAG GTG CCG TTC GAG TCC ATC CCG CTC CCT GCG TGC CCG CCG GCG GTG AAC CCC GTG GTG TCC TAC GCG GTG GCT CTC ACC TGT CAA TGT

Asp Val Arg Phe Glu Ser Ile Arg Leu Pro Gly Cys Pro Arg Gly Val Asn Pro Val Val Ser Tyr Ala Val Ala Leu Ser Cys Glu Cys

GCA CTC TGC CCG CCG ACC ACC ACT GAC TGC GCG GGT CCC AAG GAC CAC CCC TTG ACC TGT GAT GAC CCC CCG TTC CAG GAG TCC TCT TCC

Ala Leu Cys Arg Arg Ser Thr Thr Asp Cys Gly Gly Pro Lys Asp His Pro Leu Thr Cys Asp Asp Pro Arg Phe Glu Asp Ser Ser Ser

TCA AAG GCG CTT CCC CCC ACC CTT CCA AGC CCA TCC CCA CTC CCG GCG CCC TCG GAG ACC CCG ATC CTC CCA CAA TAA GAACTCCCTGAG

Ser Lys Ala Pro Pro Pro Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro Glu ... BamHI XbaI

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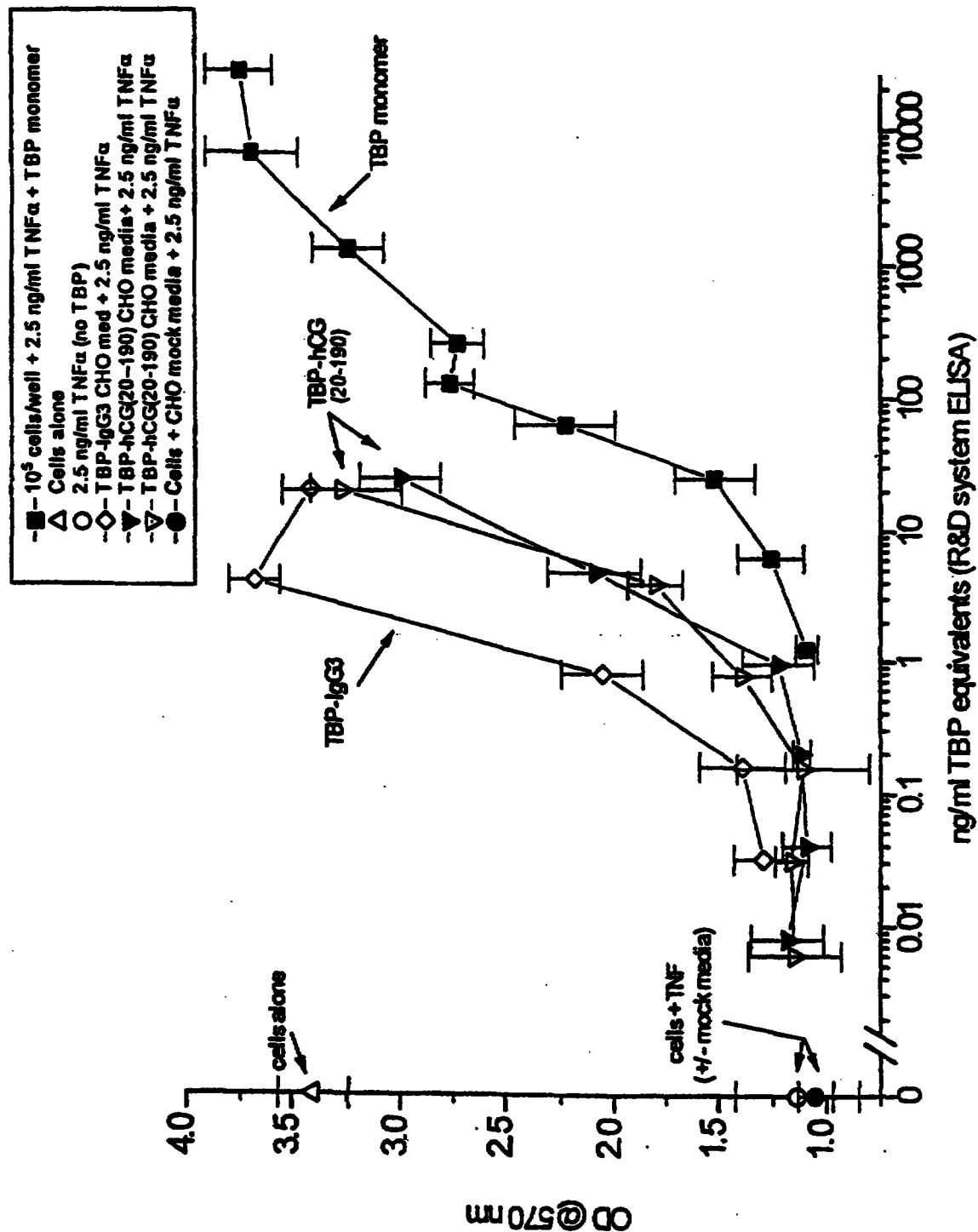


Figure 2 (b)
TBP(20-190)-hCGβ FUSION CONSTRUCT

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Figure 4. CHO cell expressed TBP-hCG(20-190) inhibits TNF α -induced cytotoxicity on BT-20 cells



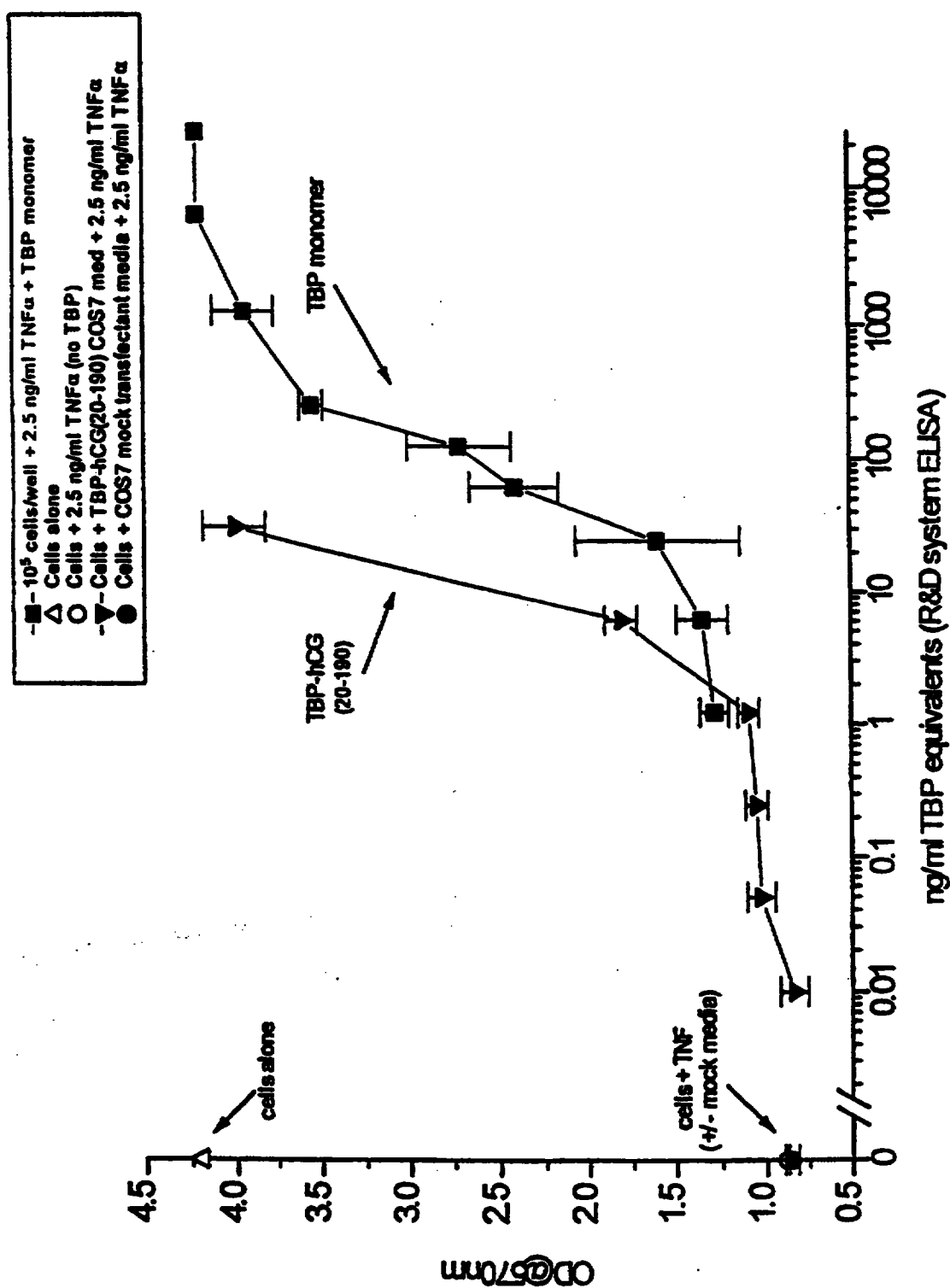
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Figure 5: COS cell expressed TBP-hCG(20-190) inhibits TNF α -induced cytotoxicity on BT-20 cells



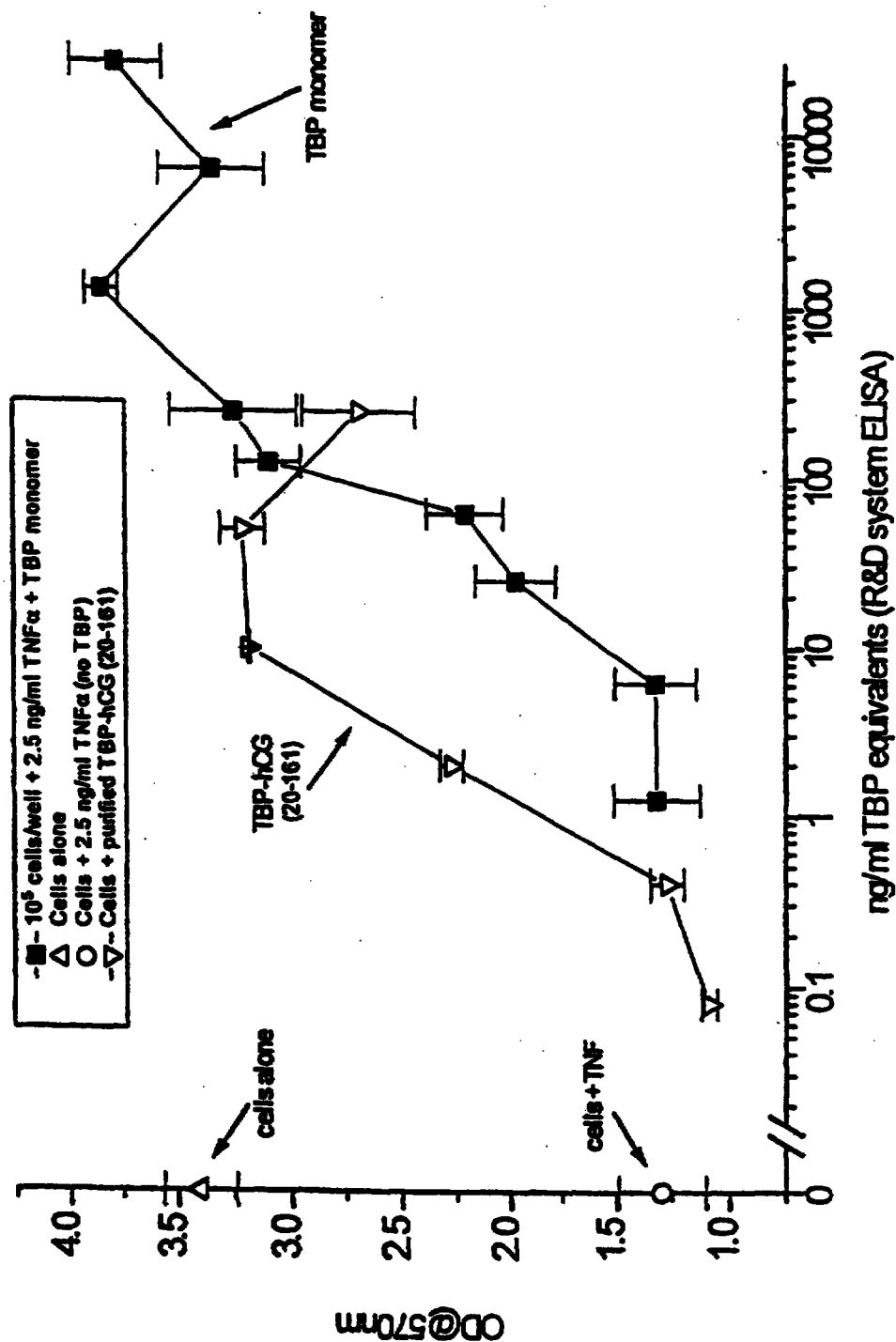
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Figure 6. Affinity purified CHO cell expressed TBP-hCG(20-161) inhibits TNF α -induced cytotoxicity on BT-20 cells



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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: C12N 15/62, 15/16, C07K 14/59, 14/715, 14/72, 16/46, C12N 15/85, 5/10, A61K 38/24	A1	(11) International Publication Number: WO 97/30161 (43) International Publication Date: 21 August 1997 (21.08.97)
(21) International Application Number: PCT/US97/02315 (22) International Filing Date: 20 February 1997 (20.02.97) (30) Priority Data: 60/011,936 20 February 1996 (20.02.96) US (60) Parent Application or Grant (63) Related by Continuation US 60/011,936 (CIP) Filed on 20 February 1996 (20.02.96) (71) Applicant (for all designated States except US): APPLIED RESEARCH SYSTEMS ARS HOLDING N.V. [NL/NL]; 14 John B. Gorsiraweg, Curacao (AN). (72) Inventors; and (75) Inventors/Applicants (for US only): CAMPBELL, Robert K. [US/US]; 25 Meadowbrook Drive, Wrentham, MA 02093 (US). JAMESON, Bradford A. [US/US]; 76 Robbins Street, Milton, MA 02186 (US). CHAPPEL, Scott C. [US/US]; 125 Canton Avenue, Milton, MA 02186 (US).		(74) Agent: BROWDY, Roger, L.; Browdy and Neimark, P.L.L.C., Suite 300, 419 Seventh Street N.W., Washington, DC 20004 (US). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.
(54) Title: HYBRID PROTEINS WHICH FORM HETERODIMERS (57) Abstract A hybrid protein includes two coexpressed amino acid sequences forming a dimer. Each sequence contains the binding portion of a receptor, such as TBP1 or TBP2, or a ligand, such as IL-6, IFN- β and TPO, linked to a subunit of a heterodimeric proteinaceous hormone, such as hCG. Each coexpressed sequence contains a corresponding hormone subunit so as to form a heterodimer upon expression. Corresponding DNA molecules, expression vectors and host cells are also disclosed as are pharmaceutical compositions and a method of producing such proteins.		

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Figure 3 is a graph illustrating the dose dependent protective effect of CHO cell expressed TBP-hCG(20-190) on TNF α -induced cytotoxicity on BT-20 cells and various controls.

Figure 4 is a graph illustrating the dose dependent protective effect of COS cell expressed TBP-hCG(20-190) on TNF α -induced cytotoxicity on BT-20 cells and various controls.

Figure 5 is a graph illustrating the dose dependent protective effect of affinity purified CHO cell expressed TBP-hCG(20-161) on TNF α -induced cytotoxicity on BT-20 cells and various controls.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention.

EXAMPLES

Materials and Methods

Cell lines used in this study were obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, unless otherwise specified. The CHO-DUKX cell line was obtained from L. Chasin at Columbia University through D. Houseman at MIT (39). The CHO-DUKX cells, which lack a functional gene for dihydrofolate reductase, were routinely maintained in complete α -plus Modified Eagles Medium (α (+)MEM) supplemented with 10% fetal bovine serum (FBS). The COS-7 cells were routinely maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS. Unless specified otherwise, cells were split to maintain them in log phase of growth, and culture reagents were obtained from GIBCO (Grand Island, New York).

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TNF α added was determined earlier in titration studies, and represents a dose at which about 50% of the cells are killed.

After addition of the samples, the cells are cultured for 48 hours at 39°C, after which the proportion of live cells is determined using crystal violet staining and a microtiter plate reader (570 nm).

RESULTS

1. Constructs under study

The designs of the hybrid proteins studied are briefly summarized below; two control proteins, a monomeric soluble p55 (r-hTBP-1) and a dimeric TBP-immunoglobulin fusion protein (TBP-IgG3) (prepared essentially as described in (10)), were studied for comparative purposes.

	<u>Construct</u>	<u>TBP N-term</u>	<u>TBP C-term</u>	<u>Fusion partner</u>
	r-hTBP-1	mix of 9 and 20	180	none
20	TBP-IgG3	mix of 9 and 20	190	IgG3 heavy chain constant region
	TBP(20-161)-hCG	20	161	hCG α and hCG β (heterodimer)
25	TBP(20-190)-hCG	20	190	hCG α and hCG β (heterodimer)

The sequences of the DNAs encoding, TBP(20-190)-hCG and TBP(20-161)-hCG are provided in Figures 1 and 2, respectively.

2. Secretion of TBP-hCG proteins

All of the constructs tested were found to be produced and secreted into culture media by transfected mammalian cells. Data illustrating this are shown in Tables 1 and 2.

3. TBP-hCG(α/β) fusion proteins assemble into heterodimers

The combination of TBP-hCG α and TBP-hCG β was confirmed using the sandwich assay for the hCG heterodimer.

Only the combined transfection of α and β subunit fusions resulted in heterodimer detection (Table 3).

4. TBP-hCG hybrid proteins exhibit increased activity over TBP monomer

Hybrid proteins produced in either COS-7 or CHO cells were found to be potent inhibitors of TNF α in the BT-20 bioassay. Some of the samples tested are summarized in Table 4.

Negative controls (conditioned media from mock transfections) were included for the 1x media samples.

As illustrated in Figures 3-5 (points on y-axis), addition of TNF (2.5 ng/ml) results in a clear reduction in live cell number (as assessed by OD 570). In every case, active samples have as a maximal protective effect the restoration of cell viability to the level seen in the absence of added TNF (i.e., the control labeled "cells alone").

The positive controls, r-hTBP-1 and TBP-IgG3, are both protective, showing a clear dose-dependence and ED50s of approximately 100 ng/ml for the r-hTBP-1 (Figs. 3-5) and about 1.5 ng/ml for TBP-IgG3 (Fig. 3) respectively.

The TBP-hCG constructs from 1x media (CHO or COS) or from the immunopurification show dose-dependent protection, with approximate ED50s ranging from 2-11 ng/ml (Figs. 3-5).

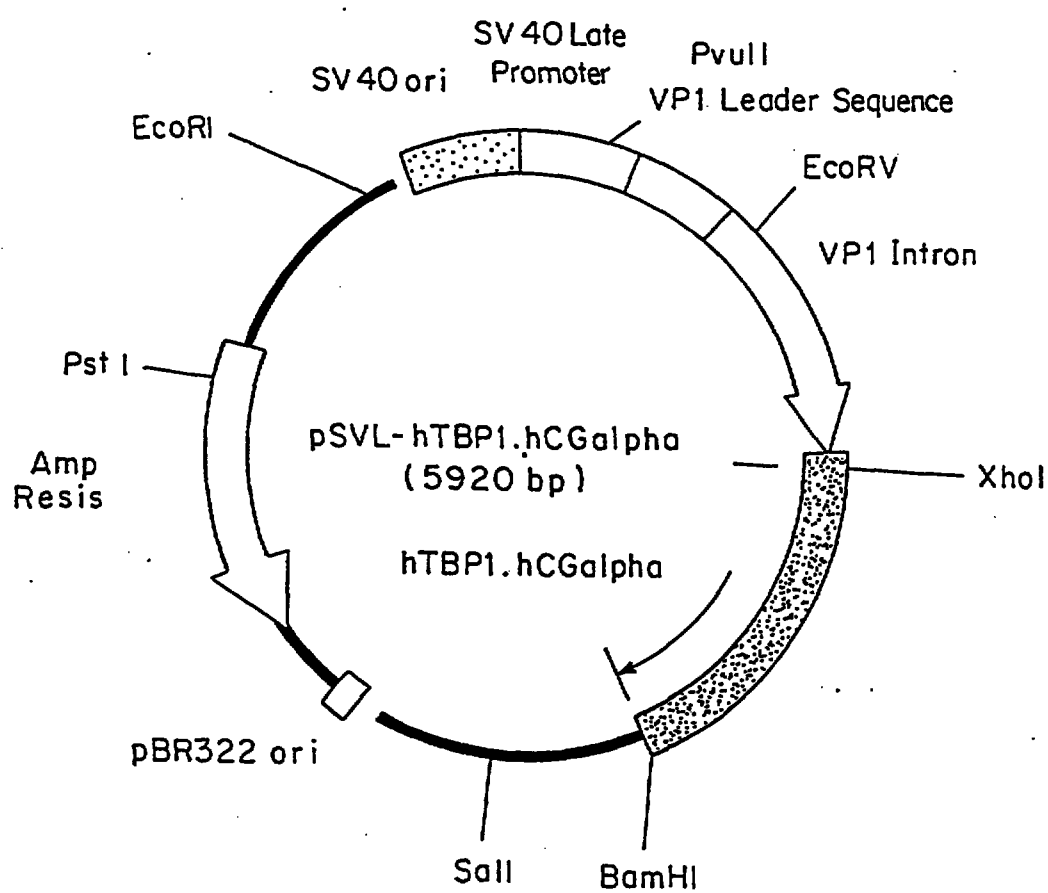
The results from the *in vitro* bioassay are reported in Table 5. The data indicate that the hybrid proteins inhibit TNF cytotoxicity, and that they are substantially more potent than the TBP monomer. The negative controls were devoid of protective activity.

In addition to the possibility that dimerization of TBP may increase potency, it is also possible that the activity of the hybrid proteins are not related to dimeric interaction with TBP, but rather to steric inhibition due to the partner of the hybrid interfering with soluble TBP/TNF binding to cell-surface TNF receptors.

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference

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FIG. 1a(1)



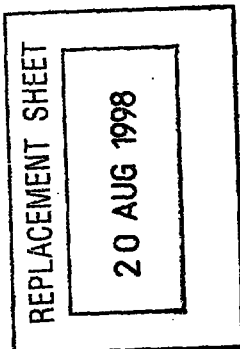


FIG. 1a(2)

Xho I hGH Signal Sequence

TCGAG ATG GCT ACA G GTAAGCGCCCTAAATCCCTTTGGGCACAAATGTCTCGAGGAGAGGACGACCTGTAGATGGACGGGGGCACCTAACCCCTCAGGTTTGGGGTTTCT
Met Ala Thr

hGH Intron

GAATGTGAGTATCGCCCATGTAAAGCCCGATATTGGCCCAATCTCAGAAAGCTCTCTGGTCCCTGGAGGATGGAGAGAGAAAACAACAGCTCCTGGAGCAGGGAGAGTGTGGCCCTCTTGTCTTC

CGGCTCCCTCTGTGGCCCTCTGTGGTTTCTCCCGAGGC TCC CGG ACG TCC CTG CTG GCT TTT GGC CTG CTG TGC CTG CCC TGG CTT
Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Cys Leu Pro Trp Leu

+20 Asp of Processed TBP1

CAA GAG GGC AGT GCC GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC AAG TGC CAC AAA AAA
Gln Glu Gly Ser Ala Asp Ser Val Cys Pro Gln Gly Tyr Ile His Pro Gln Asn Ser Ile Cys Cys Thr Lys Cys His Lys Gly

ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CAC CTC
Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg Glu Cys Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu

AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TGC ACA GTG GAC CGG GAC ACC GTG TGT GGT GGC TGC
Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys

AGG AAG AAC CAG TAC CCG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC AAT GGG ACC GTG CAC CTC TCC TGT
Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys Ser Leu Asn Gly Thr Val His Leu Ser Cys

Linker

CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC CAT GCA GGT TTC CTA AGA GAA AAC GAG TGT GTC TCC TGT GCC GGT GCT GCC CCA GGT
Gln Glu Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Leu Arg Glu Asn Glu Cys Val ser Cys Ala Gly Ala Ala Pro Gly

+7 Cys of hCG alpha

TGC CCA GAA TGC ACG CTA CAG GAA AAC CCA TTC TCC CAG CCG GGT GCC CCA ATA CTT CAG TGC ATG GGC TGC TGC TTC TCT AGA GCA TAT
Cys pro Glu Cys Thr Leu Gln Glu Asn Pro Phe Ser Gln Pro Gly Ala Pro Ile Leu Gln Cys Met Gly Cys Cys Phe Ser Arg Ala Tyr

CCC ACT CCA CTA AGG TCC AAG AAG ACG ATG TTG GTC CAA AAG AAC GTC ACT TCA GAG TCC ACT TGC TGT GTA GCT AAA TCA TAT AAC AGG GTC
Pro Thr Pro Leu Arg Ser Lys Lys Thr Met Leu Val Gln Lys Asn Val Thr Ser Glu Ser Thr Cys Cys Val Ala Lys Ser Tyr Asn Arg Val

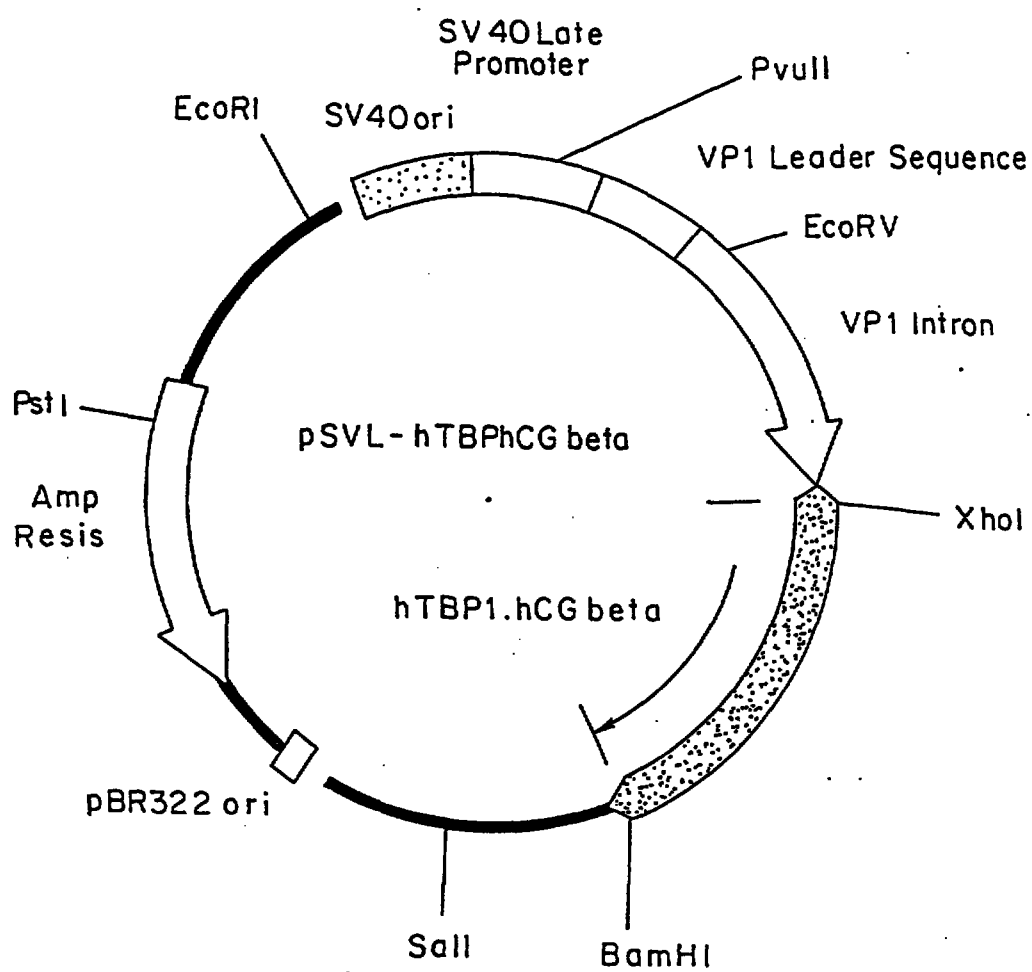
ACA GTA ATG GGG GGT TTC AAA GTG GAG AAC CAC ACG GCG TGC CAC TGC AGT ACT TGT TAT CAC AAA TCT TAA G
Thr Val Met Gly Gly Phe Lys Val Glu Asn His Thr Ala Cys His Cys Ser Thr Cys Tyr Tyr His Lys Ser ... |

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FIG. 1b(1)



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FIG. 1b(2)

hGH Signal Sequence

hGH Intron

XhoI

CTCGAG ATG GCT ACA G GTAAGCGCCCTAATATCCCTTTGGGCACATGTGTCTCTAGGGGAGAGGTAGCGACCTGTAGATGGACGGGGGCACCTAACCCCTCAGGTTTGGG
 ▶ Met Ala Thr

GCTTCTGATGTGAGTATCGCCATGTAAGCCACGATATTGGCCATCTCAGAAAGCTCTCTGGTGGGATGGAGAGAGAAAACAAACAGCTCTGGAGCGGAGAGTGCTGAC

CTCTTGCTCTCCGGCTCCCTCTGTGTCCTCTGTGTTCTCTCCAGGC TCC CGG ACG TCC CTG CTC CTG GCT TTT GGC CTG CTC TGC CTG
 ▶ Ser Arg Thr Ser Leu Leu Ala Phe Gly Leu Leu Cys Leu

+20 Asp of Processed TBPI

CCC TGG CTT CAA GAG GGC AGT GCC GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC
 ▶ Pro Trp Leu Gln Glu Gly Ser Ala Asp Ser Val Cys pro Gln Gly Lys Tyr Ile His pro Gln Asn Ser Ile Cys Cys Thr

AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC
 ▶ Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys pro Gly pro Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr

GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC
 ▶ Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp

CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CCG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TSC AGC CTC TGC CTC
 ▶ Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu

AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAT GAG TGT GTC
 ▶ Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Leu Arg Glu Asn Glu Cys Val

+7 Pro of hCG beta

Linker

TCC TGT GCT GGT GGT CCA CGG TGC CCC ATC AAT GCC ACC CTG GCT GTG GAG AAG GAG GGC TGC CCC GTG TGC ATC ACC GTC
 ▶ Ser Cys Ala Gly Ala Gly Pro Arg Cys Arg Pro Ile Asn Ala Thr Leu Ala Val Glu Lys Glu Gly Cys Pro Val Cys Ile Thr Val

AAC ACC AAC ATC TGT GCC TAC TGC CCC ACC ATG ACC CGC GTG CTG CAG GGG GTC CTG CCG GCC CTG CCT CAG GTG GTG TGC AAC TAC
 ▶ Asn Thr Thr Ile Cys Ala Gly Tyr Cys pro Thr Met Thr Arg Val Leu Leu Gln Gly Val Leu pro Ala Leu pro Gln Val Val Cys Asn Tyr

CGG GAT GTG CGC TTC GAG TCC ATC CGG CTC CCT GGC TGC CCG GGC GTG AAC CCC GTG GTC TCC TAC GCC GTG GCT CTC AGC TGT CAA
 ▶ Arg Asp Val Arg Phe Glu Ser Ile Arg Leu pro Gly Cys pro Arg Gly val Asn pro Val Ser Tyr Ala Val Ala Leu Ser Cys Gln

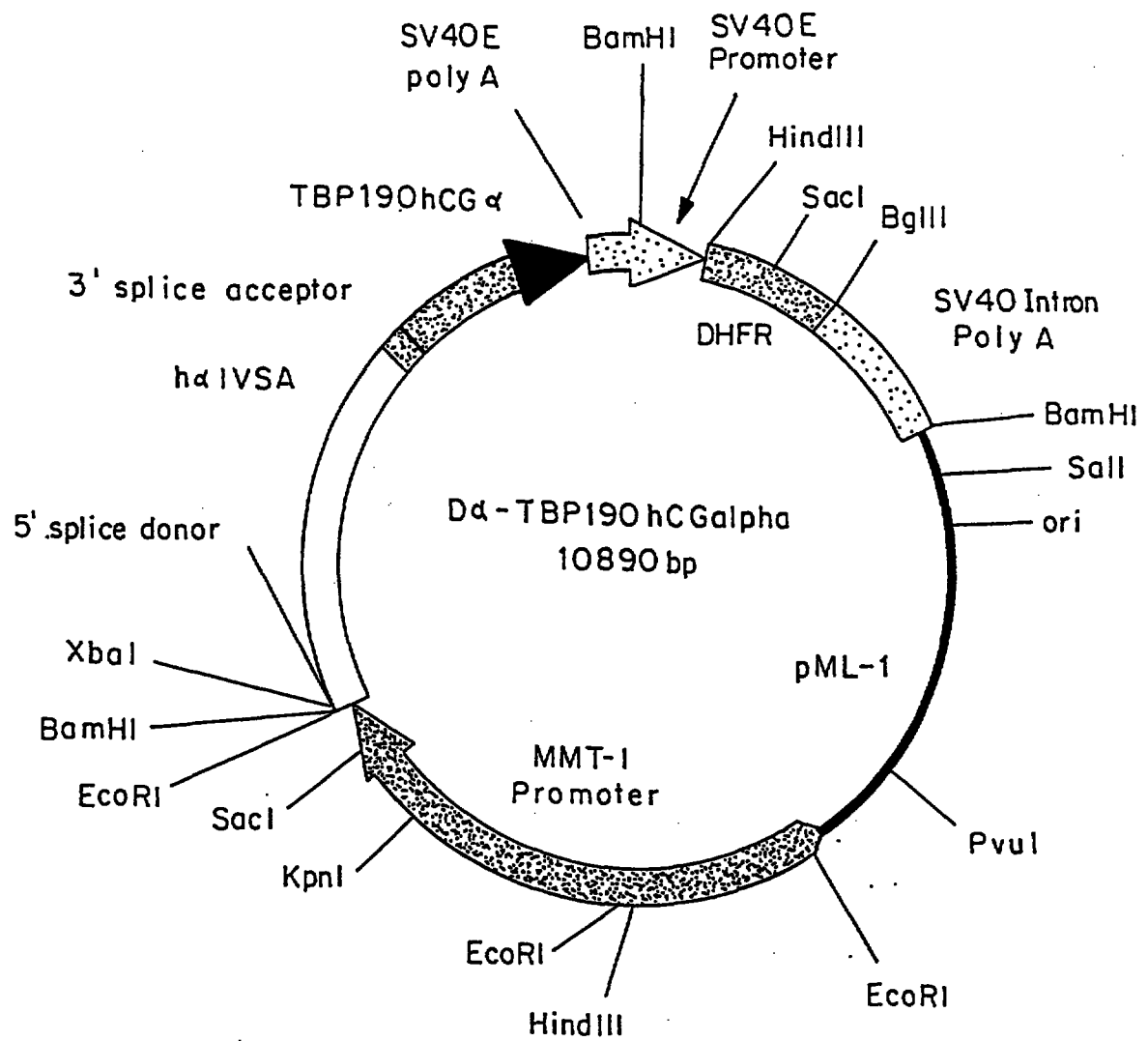
TGT GCA CTC TGC CGC AGC ACC ACT GAC TGC GGG GGT CCC AAG GAC CAC CCC TTG ACC TGT GAT GAC CCC CGC TTC CAG GAC TCC TCT
 ▶ Cys Ala Leu Cys Arg Ser Thr Thr Asp Cys Gly Gly pro Lys Asp His pro Leu Thr Cys Asp pro Arg Phe Gln Asp Ser Ser

TCC TCA AAG GCC CCT CCC AGC CTT CCA AGC CCA TCC CGA CTC CCG GGG CCC TCG GAC ACC CCG ATC CTC CCA CAA TAA
 ▶ Ser Ser Lys Ala pro pro Ser Leu pro Ser pro Arg Leu pro Gly pro Ser Asp Thr pro Ile Leu pro Gln ***

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FIG. 2a(1)



XhoI
 TCGAG ATG GCT ACA G
 hGH Signal Sequence
 hGH Intron
 G TGAAGCGCCCTAAATAATCCCTTTGGGGCAAAATGTGTCTGAGGGAGAGGGCAGCCTGTAGATGGGACGGGGGCACTAACCTCAGGTTTGGGGTTTCT

XhoI
 TCGAG ATG GCT ACA G
 hGH Signal Sequence
 hGH Intron
 G TGAAGCGCCCTAAATAATCCCTTTGGGGCAAAATGTGTCTGAGGGAGAGGGCAGCCTGTAGATGGGACGGGGGCACTAACCTCAGGTTTGGGGTTTCT

CGAATGTGAGTATCGCCATGTAAGCCACGATTTGGGCAATCTCAGAAAGCTCCTGGTCCCTCGAGGGATGGAGGAGAGAAACAAACAGCTCCTGGAGCAGGGAGAGTGCTGGCCCTCTTGTCTTC

CGGCTCCCTCTGTTGCCCTCTGGTTTCGCCACGGC
TCC CGG ACG TCC CTG CTC CTG GGC CTG CTC TGC CTG CCC TGG CTTT
▶ Ser Arg Thr Ser Leu Leu Ala Phe Gly Leu Cys Leu Pro Trp Leu

+20 Asp of processed TBP1

CAA GAG GGC AGT GCC GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC AAG TGC CAC AAA GGA
.....
gln glu gly ser ala asp ser val cys pro gln gly lys tyr ile his pro gln asn ser ile cys cys thr lys cys his lys gly

ACC TAC TTG TAC AAT GAC CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC ACC GGT TCA GAA AAC CAC CTC
 Thr Tyr Leu Tyr Asn Asp Cys pro Gly pro Gly Gln Asp Thr Asp Cys Arg Gly Glu Cys Thr Ala Ser Glu Asn His Leu

AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC CGG GAC ACC GTG TGT GGC TGC
 → ARG HIS CYS LEU SER CYS SER IVS CYS ARG IVS GLU MET GLY GLN VAL GLU ILE SER SER CYS THR VAL ASP ARG ASP THR VAL CYS GLY CYS

AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TAT TTC AAT TGC AGC CTC AAT GGG ACC GTG CAC CTC TCC TGC

CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC CAG GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC TCC TGT AGT AAC TGT AAG AAA AGC CTG
 Gln Glu Lys Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Leu Arg Glu Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu
 Linker +7 Cys of hCG alpha

GAG TGC ACG AAG TTG TGC CTA CCC CAG ATT GAG AAT GTT AAG GGC ACT GAG GAC TCA GGC ACC ACA GCC GGT GGC CCA GGT TGC CCA
Glu Cys Thr Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser Gly Thr Thr Ala Gly Ala Pro Gly Cys Pro

GAA TGC ACG CTA CAG GAA AAC CCA TTC TTC TCC CAG CCG GGT GCC CCA ATA CTT CAG TGC ATG GGC TGC TGC TCT AGA GCA TAT CCC ACT

CCA CTA AGG TCC AAG AAG ACG ATG TTG GTC CAA AAG AAC GTC ACC TCA GAG TCC ACT TGC TGT GTA GCT AAA TAT AAC AGG GTC ACA GTA

ATG GGG GGT TTC AAA GTG GAG AAC CAC ACG GCG TGC CAC TGC AGT ACT TGT TAT TAT CAC AAA TCT TAA GGATCCCTCGAG

Bam III Xhol

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REPLACEMENT SHEET

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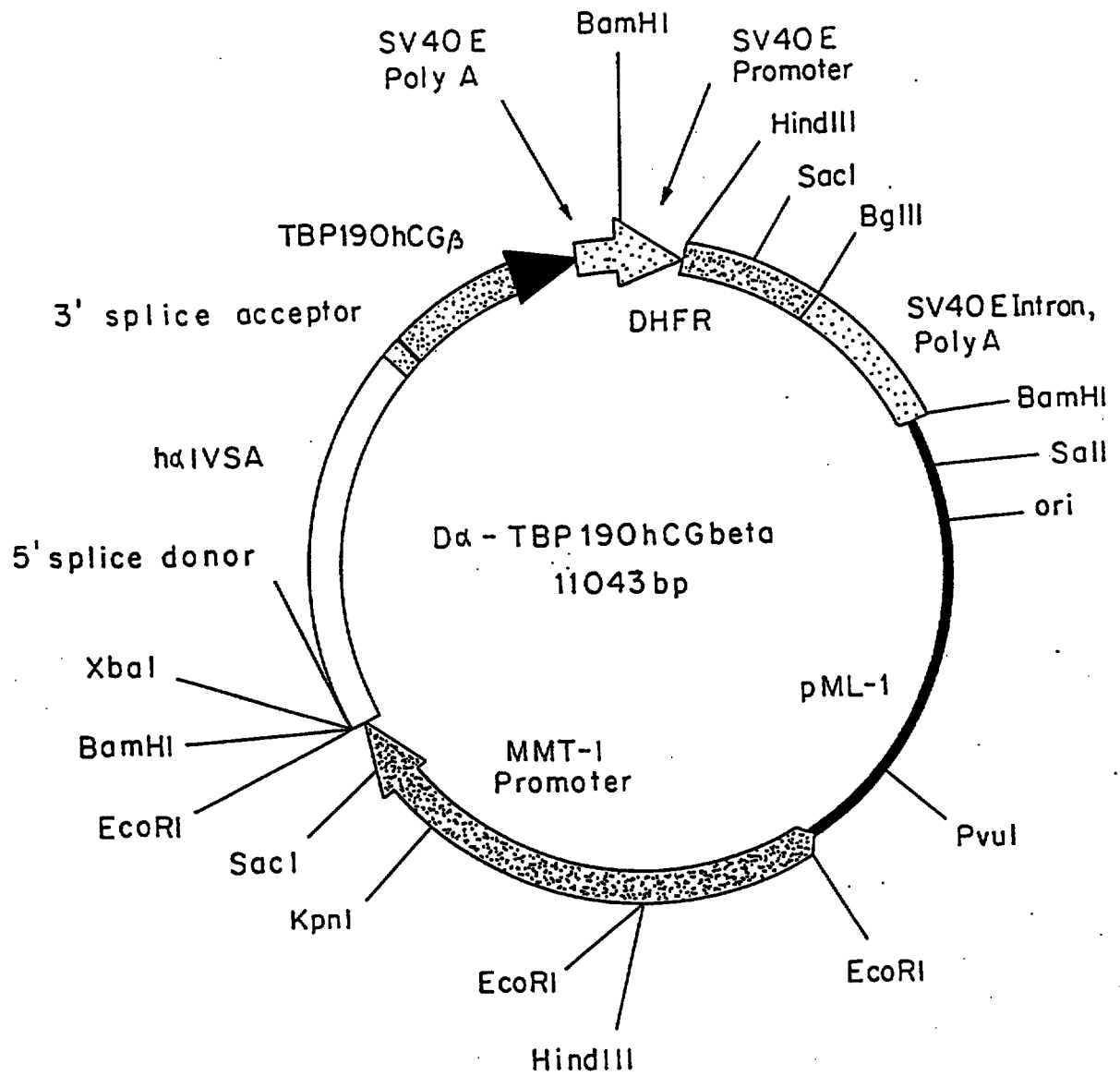
REPLACEMENT SHEET

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REPLACEMENT SHEET

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FIG. 2b(1)



REPLACEMENT SHEET

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FIG. 2b(2)

Xhol hGH Signal Sequence hGH Intrin

CTCGAG ATG GCT ACA G GTAAGCGCCCTTAATCCCTTTGGGCACATGTCTCTGGGGAGACGACGACCTGTAGATGGACGGGGCACTAACCTCCTCAGTTTGGG

► Met Ala Thr

GCTTCTGAATGTGAGTATGCGCCATGTAAGCCCGAGTATTGGCCCAATCTCAGAAAGCTCCTGGTCCCTGGAGGATGGAGAGAAACAAACAGCTCCTGTAGCAGGTAGCTGCTGGC

CTCTGTCTCTCGGCTCCTCTGTGTCCTCTGTCCTCTGTTCTCTCCCGAGG C TCC CGG ACG TCC CTG CTC CTG GCT TTT GGC CTG CTG CTG CTG

► Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu Cys Leu

+20 Asp of Processed TBPI

CCC TGG CTT CAA GAG GGC AGT GCC GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC

► Pro Trp Leu Gln Glu Gly Ser Ala Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Ser Ile Cys Cys Thr

AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC

► Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Asp Thr Asp Cys Arg Glu Cys Gly Ser Phe Thr

GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC

► Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp

CGG GAC ACC GTG TGT GGC TGC ACC GAG AAA CAG AAG AAC CAG TAC TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC

► Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Ser Leu Cys Leu

AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC

► Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Leu Arg Glu Asn Glu Cys Val

TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG TGC CTA CCC CAG ATT GAG AAT GTT AAG GGC ACT GAG GAC TCA GGC ACC

► Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser Gly Thr

Linker

+7 Pro of beta

ACA GCT GGT GCT GGT CCA CGG TGC CGC CCC ATC AAT GCC ACC CTG GGT GAG AAG GAG GGC TGC CCC GTG TGC ATC ACC GTC AAC

► Thr Ala Gly Ala Gly Pro Arg Cys Arg Pro Ile Asn Ala Thr Leu Ala Val Glu Lys Glu Gly Cys Pro Val Cys Ile Thr Val Asn

ACC ACC ATC TGT GCC GGC TAC TGC CCC ACC ATG ACC CGC GTG CTG CAG GGC GTG CCC GCG CTG CCT CAG GTG GTG TGC AAC TAC CGC

► Thr Thr Ile Cys Ala Gly Tyr Cys Pro Thr Met Thr Arg Val Leu Leu Gln Gly Val Leu Pro Ala Leu Pro Gln Val Cys Asn Tyr Arg

GAT GTG CGC TTC GAG TCC ATC CGG CTC CCT GGC TGC CCG CGC GTG AAC CCC GTG TCC TAC GCC GTG GCT CTC AGC TGT CAA TGT

► Asp Val Arg Phe Glu Ser Ile Arg Leu Pro Gly Cys Pro Arg Gly Val Asn Pro Val Val Ser Tyr Ala Val Ala Leu Ser Cys Gln Cys

GCA CTC TGC CGC AGC ACC ACT GAC TGC GGG GGT CCC AAG GAC CAC CCC TTG ACC TGT GAT GAC CCC CGC TTC CAG GAC TCC TCT TCC

► Ala Leu Cys Arg Arg Ser Thr Thr Asp Cys Gly Gly Pro Lys Asp His Pro Leu Thr Cys Asp Asp pro Arg Phe Gln Asp Ser Ser

TCA AAG GCC CCT CCC AGC CTT CCA AGC CCA TCC CGA CTC CCG GGG CCC TCG GAC ACC CCG ATC CTC CCA TAA GGATCCCTCGAG

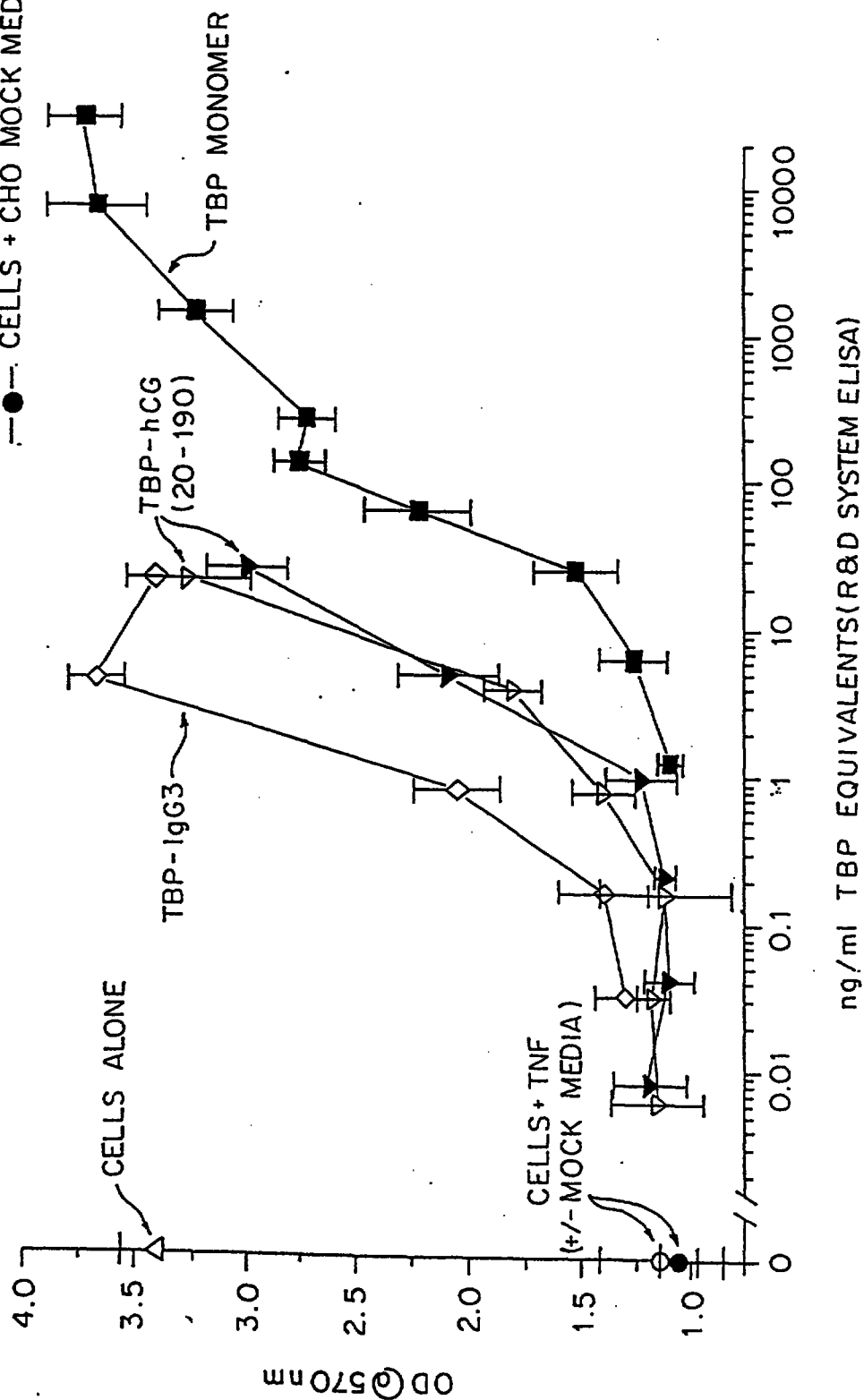
► Ser Lys Ala Pro Pro Ser Leu Pro Ser Pro Arg Leu Pro Gly pro Ser Asp Thr pro Ile Leu Pro Gln *** BamHI Xhol

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- 10⁵ CELLS/WELL + 2.5 ng/ml TNF α + TBP MONOMER
 Δ CELLS ALONE
 ○ 2.5 ng/ml TNF α (NO TBP)
 —◇— TBP-IgG3 CHO MED + 2.5 ng/ml TNF α
 —▼— TBP-hCG (20-190) CHO MEDIA + 2.5 ng/ml TNF α
 —▽— TBP-hCG (20-190) CHO MEDIA + 2.5 ng/ml TNF α
 —●— CELLS + CHO MOCK MEDIA + 2.5 ng/ml TNF α

FIG. 3



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- 10⁵ CELLS / WELL + 2.5 ng/ml TNF α + TBP MONOMER
 Δ CELLS ALONE
 O CELLS + 2.5 ng/ml TNF α (NO TBP)
 —▼— CELLS + TBP-hCG (20-190) COS7 MED + 2.5 ng/ml TNF α
 ● CELLS + COS7 MOCK TRANSFECTANT MEDIA + 2.5 ng/ml TNF α

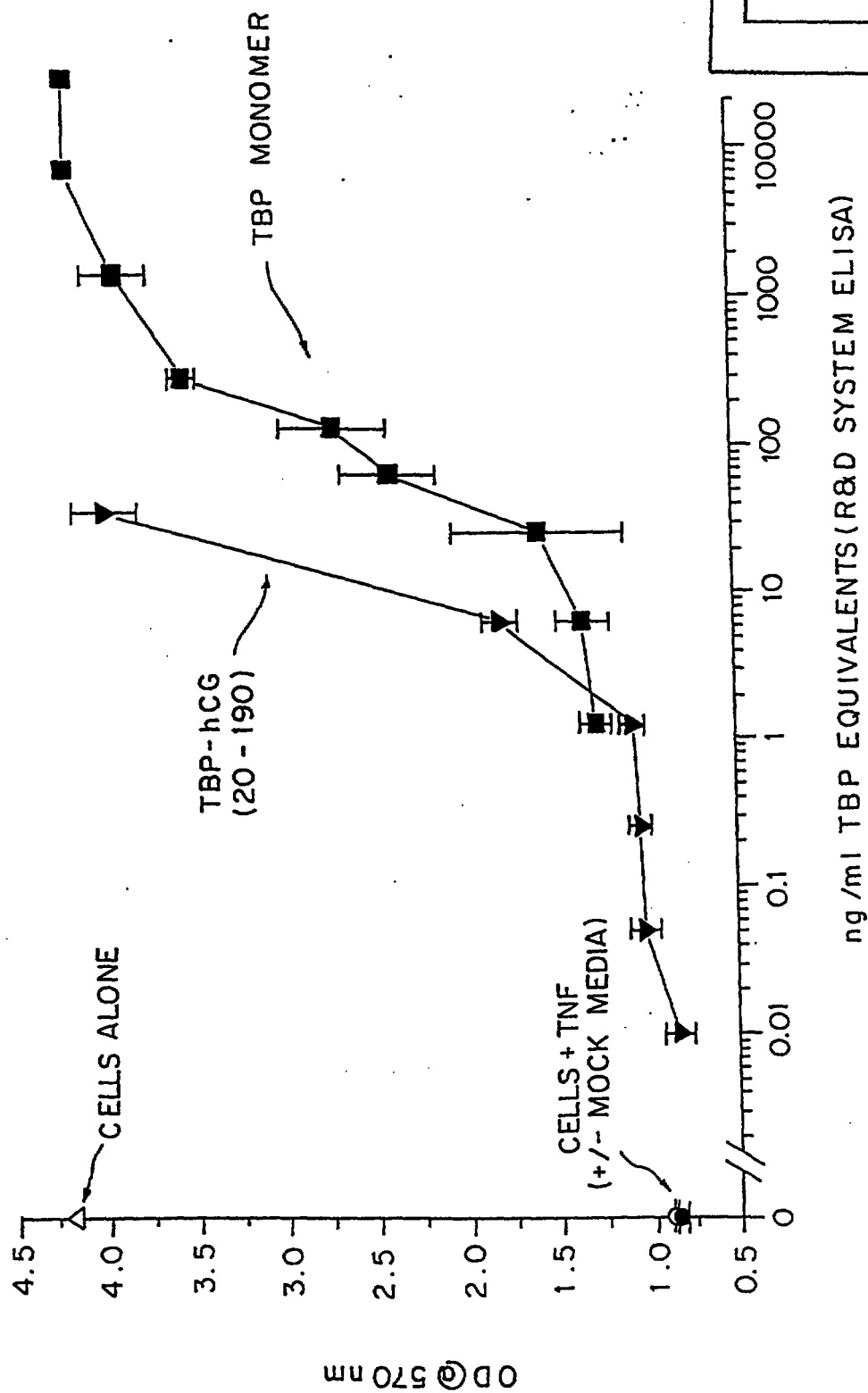


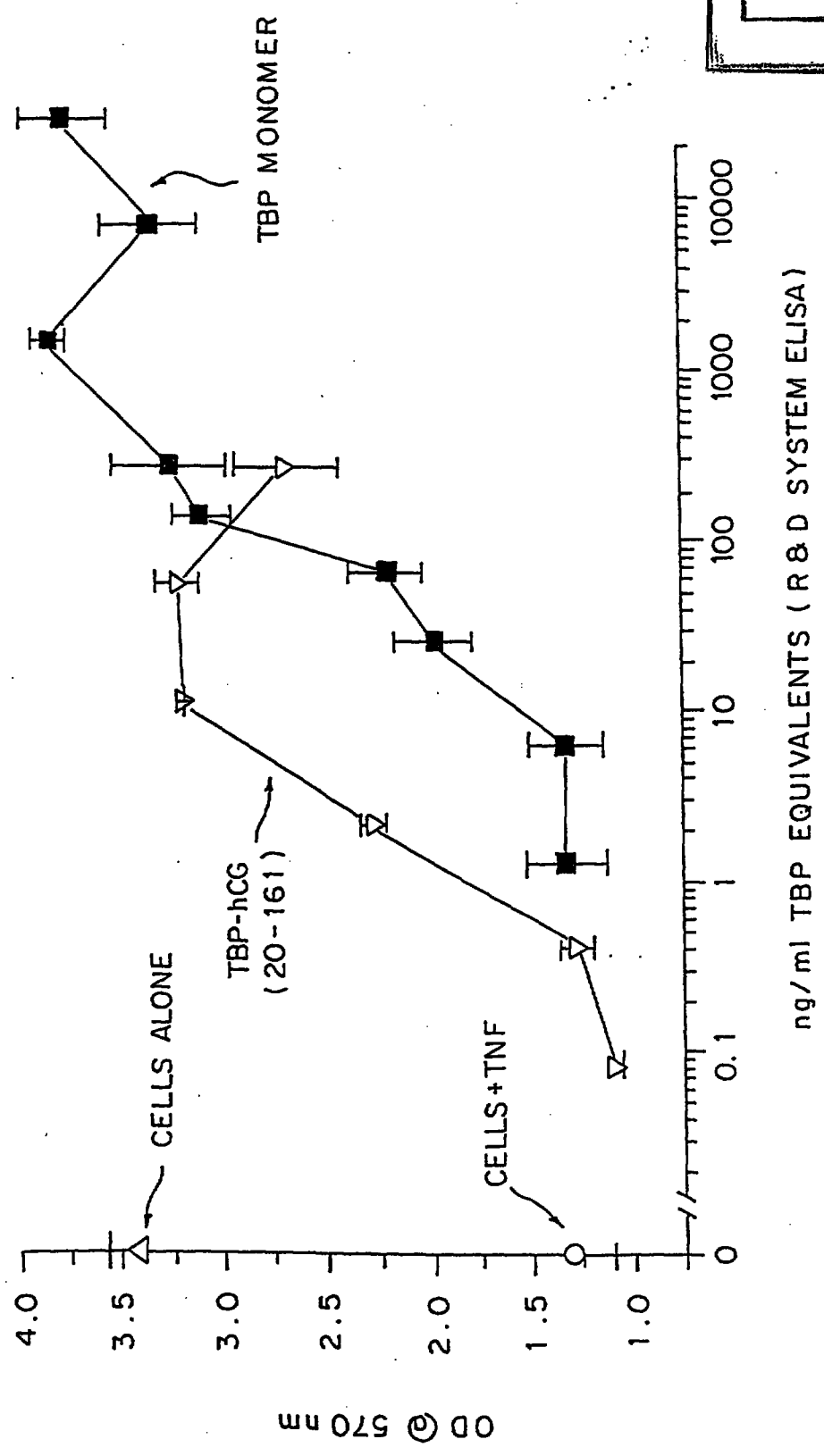
FIG. 4

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- 10⁵ CELLS / WELL + 2.5 ng/ml TNFα + TBP MONOMER
- △ CELLS ALONE
- CELLS + 2.5 ng/ml TNFα (NO TBP)
- ▽ CELLS + PURIFIED TBP-hCG (20-161)

FIG. 5





14. A hybrid protein in accordance with claim 1, wherein one or more covalent bonds between the two subunits (b) are added.

15. A DNA molecule encoding a hybrid protein in accordance with claim 1..

16. An expression vector containing a DNA molecule in accordance with claim 15.

17. A host cell containing an expression vector in accordance with claim 16 and capable of expressing said hybrid protein.

18. A method for producing hybrid protein comprising culturing a host cell in accordance with claim 17 and recovering the hybrid protein expressed thereby.

19. A pharmaceutical composition comprising a hybrid protein in accordance with claim 1 and a pharmaceutically acceptable carrier and/or excipient.

20. The use of a pharmaceutical composition comprising the hybrid protein of claim 8 for inducing follicular maturation to a subject in need thereof.

As used herein, "fusion protein" refers to a hybrid protein, which consists of two or more proteins, or fragments thereof, linked together covalently.

5 The term "aggregation", as used herein, means the formation of strong specific non-covalent interactions between two polypeptide chains forming a complex, such as those existing between the α and β subunit of a heterodimeric hormone (such as FSH, LH, hCG or TSH).

10 The terms "ligand" or "ligand protein", as used herein, refer to a molecule, other than an antibody or an immunoglobulin, capable of being bound by the ligand-binding domain of a receptor; such molecule may occur in nature, or may be chemically modified or chemically synthesised.

15 The term "ligand-binding domain", as used herein, refers to a portion of the receptor that is involved in binding a ligand and is generally a portion or essentially all of the extracellular domain.

20 The term "receptor", as used herein, refers to a membrane protein, whose binding with the respective ligand triggers secondary cellular responses that result in the activation or inhibition of intracellular process.

25 In a further aspect, the present invention provides the use of the hybrid protein as a medicament. The medicament is preferably presented in the form of a pharmaceutical composition comprising the protein of the invention together with one or more pharmaceutically acceptable carriers and/or excipients. Such pharmaceutical compositions represent yet a further aspect of the invention.

30 BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be better understood by reference to the appended drawings, in which:

35 Figures 1(a) and 1(b) show the TBP(20-161)-hCG α and TBP(20-161)-hCG β constructs, respectively, and the corresponding sequences (SEQ ID NOS:1-4). The linker sequence shown in Fig. 1(a) is Ala-Gly-Ala-Ala-Pro-Gly (SEQ ID NO:9). The linker sequence shown in Fig. 1(b) is Ala-Gly-Ala-Gly (SEQ ID NO:10).

Figures 2(a) and 2(b) show the TBP(20-190)-hCG α and TBP(20-190)-hCG β constructs, respectively, and the corresponding sequences (SEQ ID NOS:5-8).

Figure 3 is a schematic summary of the constructs of Figures 1 and 2 showing p55 TNFR1, TBP1 and TBP1 fusion constructs. The linker sequences shown on the last two lines are SEQ ID NO:9 (Ala-Gly-Ala-Ala-Pro-Gly) and SEQ ID NO:10 (Ala-Gly-Ala-Gly).

Figure 4 is a graph illustrating the dose dependent protective effect of CHO cell expressed TBP-hCG(20-190) on TNF α -induced cytotoxicity on BT-20 cells and various controls.

Figure 5 is a graph illustrating the dose dependent protective effect of COS cell expressed TBP-hCG(20-190) on TNF α -induced cytotoxicity on BT-20 cells and various controls.

Figure 6 is a graph illustrating the dose dependent protective effect of affinity purified CHO cell expressed TBP-hCG(20-161) on TNF α -induced cytotoxicity on BT-20 cells and various controls.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention.

EXAMPLES

Materials and Methods

Cell lines used in this study were obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, unless otherwise specified. The CHO-DUKX cell line was obtained from L. Chasin at Columbia University through D. Houseman at MIT (39). The CHO-DUKX cells, which lack a functional gene for dihydrofolate reductase, were routinely maintained in complete α -plus Modified Eagles Medium (α (+)-MEM) supplemented with 10% fetal bovine serum (FBS). The COS-7 cells were routinely maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS. Unless specified otherwise, cells were split to maintain them in log phase of growth, and culture reagents were obtained from GIBCO (Grand Island, New York).